The Journal of Laboratory and Clinical Medicine

VOL. 25 AUGUST, 1940 NO. 11

CLINICAL AND EXPERIMENTAL

EXPERIMENTAL STUDY OF THE EFFECTS OF SULFAPYRIDINE ON STAPHYLOCOCCI AND STAPHYLOCOCCUS TOXIN*

R. H. RIGDON, M.D., AND P. R. FREEMAN, MEMPHIS, TENN.

THERE are only a few observations on either clinical or experimental use of sulfapyridine in staphylococcus infections.[1-3] Whitby[2] and Long[3] have found that this drug is an active chemotherapeutic agent in the treatment of staphylococcus infections in mice. The mode of action of sulfapyridine on staphylococci is not clearly understood.

This paper is a report of a series of experiments in which the in vitro action of sulfapyridine† was studied on three strains of staphylococci. It also includes a study of the action of sulfapyridine on staphylococcus toxin both in vitro and in vivo.

EFFECT OF SULFAPYRIDINE ON STAPHYLOCOCCI IN VITRO

Three strains of staphylococci were used in this experiment. Strain "A-O" is a variant from a culture of *Staphylococcus aureus,* isolated in 1933 from the pharynx of a patient with agranulocytic angina. It produces a yellow pigment, a toxin that lyses rabbit red blood cells, necrotizes rabbit skin, and is lethal for mice.

Strain "Wood" was obtained from Lederle Laboratories in 1937. It also produces a potent toxin that lyses red blood cells, necrotizes the skin of rabbits, and is lethal for mice. This strain of staphylococcus is hemolytic but does not produce pigment.

*From the University of Tennessee, Department of Pathology, Memphis.
Aided by a special grant from the University of Tennessee.
Received for publication, September 22, 1939.
†The sulfapyridine was obtained through the courtesy of Lederle Laboratories.

Strain "7" was isolated from a furuncle in 1937. It is nonpigmented and does not produce any hemolysis when grown on blood agar plates containing rabbit red blood cells. This strain of staphylococci does not produce toxin.

These staphylococci were grown on nutrient broth medium (Difco) with 2 per cent dextrose. It was buffered, and the pH was adjusted to 7.2. Varying amounts of a 2 per cent solution of sulfapyridine in broth were added to a series of tubes containing this medium. The total volume of broth and sulfapyridine was 5.0 c.c. The tubes were autoclaved for twenty minutes at 20 pounds pressure. The following amount of sulfapyridine was put into each tube:

SERIES	QUANTITY OF SULFAPYRIDINE	MG./C.C.
1	1.0 c.c. of 2.0 per cent solution	4.0
2	0.75 c.c. of 2.0 per cent solution	3.0
3	0.50 c.c. of 2.0 per cent solution	2.0
4	0.25 c.c. of 2.0 per cent solution	1.0
5	1.0 c.c. of 0.2 per cent solution	0.4
6	0.75 c.c. of 0.2 per cent solution	0.3
7	0.50 c.c. of 0.2 per cent solution	0.2
8	0.25 c.c. of 0.2 per cent solution	0.1
9	1.0 c.c. of 0.02 per cent solution	0.04
10	0.75 c.c. of 0.02 per cent solution	0.03
11	0.50 c.c. of 0.02 per cent solution	0.02
12	0.25 c.c. of 0.02 per cent solution	0.01

The first four series of tubes showed a small quantity of sulfapyridine in the bottom of each tube after autoclaving. The quantity of sulfapyridine precipitated decreased, however, with each dilution.

Four dilutions of each of the three strains of staphylococci were used to inoculate a group of the tubes containing the sulfapyridine as follows: (A) a twenty-four-hour broth culture; (B) a 1:10,000 dilution; (C) a 1:1,000,000 dilution; (D) a 1:10,000,000 dilution of the culture used in (A). A wire loop was used to transfer the inoculum. The medium was incubated at 37.5° C., and growth was observed at twenty-four-hour intervals. The quantity of growth in each tube is indicated by pluses, the greatest by ++++ and the least by +. Tubes are marked ? in which it was questionable whether or not there was any bacterial growth. No growth is indicated by 0.

The result of this experiment is shown in Chart 1. From these data it is evident that sulfapyridine has a bacteriostatic effect on each strain of staphylococci used in this study. The greatest effect was produced on strain "7." Blood agar plates were poured with a 1:10,000,000 dilution of each strain of staphylococci, and every plate showed too many colonies to count. Other experiments in which the colonies were counted have shown, however, that the number of bacteria per unit of inoculum will not account for this variation in growth of the three strains of staphylococci in the same concentrations of sulfapyridine.

The bacterial growth was smooth in all the tubes containing broth and sulfapyridine during the first forty-eight hours. At this time some of the tubes began to show a slightly granular appearance. Strain "Wood" showed this change more than either "A-O" or "7." The number of granules progressively increased and accumulated in the bottom of the tubes. An apparent diminution in growth accompanied the formation of granules in the medium.

This granular type of growth observed with the three strains of staphylococci suggests that some change may have occurred in the organisms during the

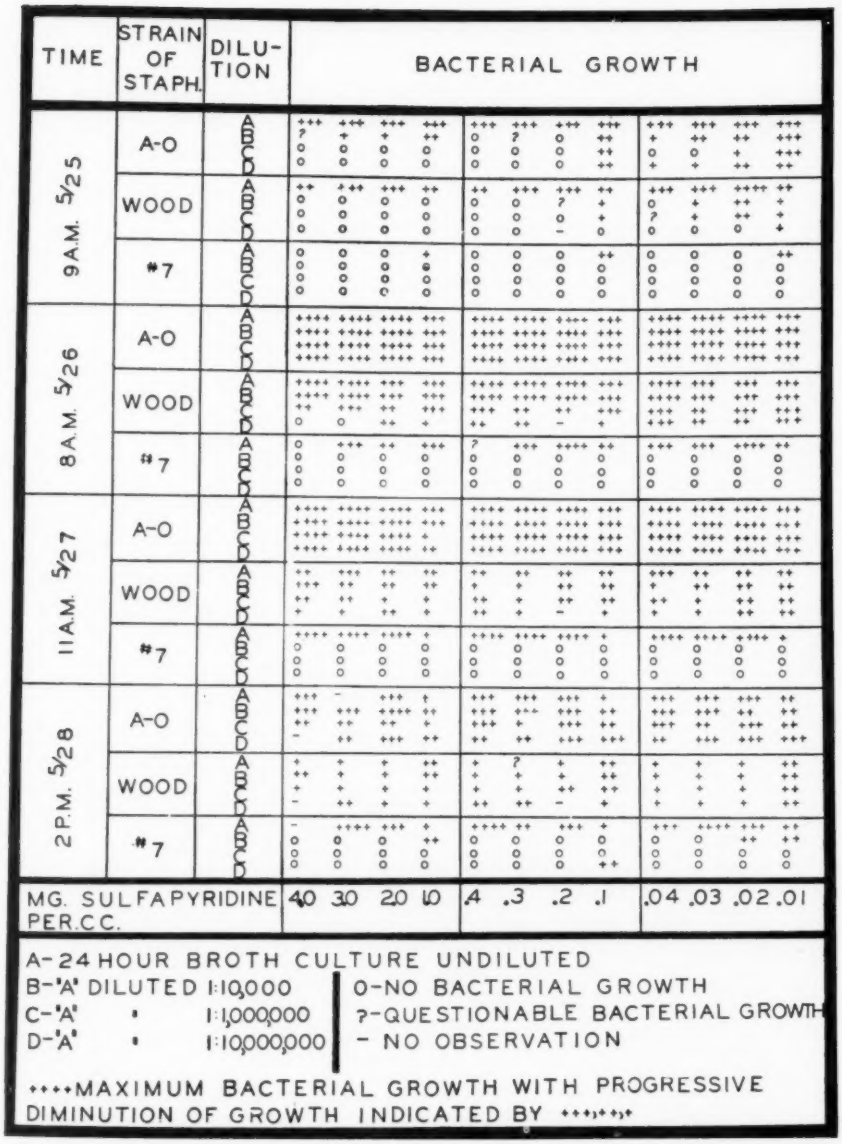


Chart 1.—The effect of different concentrations of sulfapyridine on the growth of three strains of staphylococci in vitro.

period in which they were in the presence of sulfapyridine. To determine whether the organism had been changed by the action of sulfapyridine, three groups of 14 different sugars in broth were prepared. These sugars were inoculated with each of the three strains of staphylococci which had been growing in the presence of sulfapyridine for forty-eight hours. The control inoculum was the same three cultures grown in only extract broth for twenty-four hours. The fermentation reactions after forty-eight hours were the same for the group of staphylococci grown in the presence of sulfapyridine as they were for the staphylococci in the control medium.

TABLE I

EFFECT OF SULFAPYRIDINE ON THE HEMOLYSIN IN STAPHYLOCOCCUS TOXIN

2.0 c.c. staphylococcus toxin + 100 mg. sulfapyridine	DEGREE OF LYSIS AFTER EIGHTEEN HOURS. QUANTITY OF STAPHYLOCOCCUS TOXIN, C.C.																	
	0.08	0.07	0.05	0.03	0.02	0.01	0.008	0.007	0.005	0.003	0.002	0.001	0.0008	0.0007	0.0005	0.0003	0.0002	0.0001
Sulfapyridine	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3	2
Control	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	3	3

4 = Complete lysis of red blood cells.
3 = 75 per cent lysis of red blood cells.
2 = 50 per cent lysis of red blood cells.
1 = 25 per cent lysis of red blood cells.

TABLE II

EFFECT OF SULFAPYRIDINE ON RED BLOOD CELLS IN THE PRESENCE OF STAPHYLOCOCCUS TOXIN

Quantity of sulfapyridine with rabbit red cells for 2 hr.	DEGREE OF LYSIS AFTER TWENTY-FOUR HOURS. QUANTITY OF STAPHYLOCOCCUS TOXIN, C.C.																	
	0.009	0.008	0.007	0.006	0.005	0.004	0.003	0.002	0.001	0.0009	0.0008	0.0007	0.0006	0.0005	0.0004	0.0003	0.0002	0.0001
0.1 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2	2	1	1
1 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2	2	1	1
5 mg. per c.c.	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2	2	1	1
Control	4	4	4	4	4	4	4	4	3	3	3	3	2	2	2	2	1	1

The red blood cells were suspended in saline in 1 per cent concentration.
4 = Complete lysis of red blood cells.
3 = 75 per cent lysis of red blood cells.
2 = 50 per cent lysis of red blood cells.
1 = 25 per cent lysis of red blood cells.

The failure of strain "7" to grow in a concentration of sulfapyridine in which "A-O" and "Wood" grew luxurantly, as shown in Chart 1, suggests that this chemical exerts a greater bactericidal action on one strain of staphylococci than upon another. The absence of any growth after seventy-two hours in certain tubes inoculated with strain "7" suggests that sulfapyridine may be bactericidal for certain staphylococci when the organisms are present in small numbers. Each tube inoculated with strain "7" in groups B and D, as shown in Chart 1, was cultured. The tubes were shaken, and a wire loop of inoculum was placed in extract broth. The only tubes showing a positive growth after forty-eight hours' incubation were the tubes inoculated from those showing a definite growth. These results suggest that the bacteria in these tubes apparently were dead at the time the second group of tubes was inoculated.

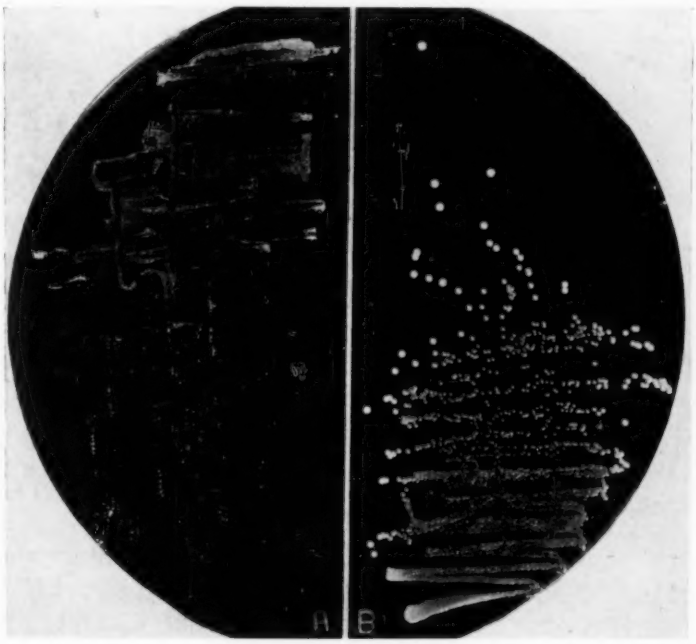


Fig. 1.—Colonies of staphylococci strain "7" on surface of blood agar plates. *A*, Plate contains 10 mg. of sulfapyridine per cubic centimeter of blood agar. *B*, Control for *A*. Photographed after twenty-four hours.

A second experiment was made to study the effect of sulfapyridine on the growth of staphylococci. In this, three different concentrations of sulfapyridine, 1, 5, and 10 mg. per c.c., were added to infusion blood agar. An equal quantity of this blood agar was then put into a group of Petri dishes. The control was blood agar with no sulfapyridine.

The control medium and the three groups of Petri dishes with sulfapyridine in the blood agar were inoculated with strains "A-O," "Wood," and "7." The plates were incubated at 37.5° C. for twenty-four hours. There was a marked diminution in the size of the colonies on the blood agar plates with 5 and 10 mg. per c.c. of sulfapyridine when compared with the controls. The

colonies from strain "7" were also much smaller than those inoculated with "A-O" and "Wood." Fig. 1 shows the colonies from strain "7" after growing on blood agar for twenty-four hours in the presence of 10 mg. per c.c. of sulfapyridine; *A* shows the bacteria growing in the presence of sulfapyridine; *B* is the control.

The amount of hemolysis that occurs around the individual colonies is decreased in those plates with sulfapyridine as compared with the controls. It is interesting to note, however, that the width of the hemolytic zone around the colonies is always proportional to the size of the colony.

EFFECT OF SULFAPYRIDINE ON HEMOLYSIS PRODUCED BY STAPHYLOCOCCUS TOXIN

In the preceding experiments it was found that hemolysis occurred around staphylococcus colonies growing in the presence of sulfapyridine. The present experiment is a further study of the effect of this drug on the hemolytic, the skin necrotizing, and the lethal factor in staphylococcus toxin.

One hundred milligrams of sulfapyridine were added to 2 c.c. of staphylococcus toxin. Only a small amount of the drug was soluble even after thorough shaking. The toxin-sulfapyridine mixture remained together for fifteen minutes and was then titrated. Washed rabbit red blood cells in a 1 per cent saline suspension were used in the hemolytic titrations. The results of this experiment are shown in Table I. From this table it can be seen that there is no difference in the degree of lysis produced by staphylococcus toxin alone and by staphylococcus toxin in the presence of sulfapyridine for fifteen minutes before titration.

To study further the action of sulfapyridine on the hemolysis produced by staphylococcus toxin, the following experiment was performed: Red blood cells from a rabbit were carefully washed four times in large volumes of saline. One cubic centimeter of these washed cells was put into each of four flasks containing 100 c.c. of saline. Sulfapyridine in quantities of 10 mg., 100 mg., and 500 mg., respectively, was added to three of the flasks; the fourth flask was the control. The red blood cells remained in the presence of the sulfapyridine for two hours, after which time 1 c.c. of the different suspensions of red blood cells was added to a series of tubes containing decreasing amounts of staphylococcus toxin. The results of this titration are shown in Table II. It is evident from these data that rabbit red blood cells treated with various concentrations of sulfapyridine for two hours react to the hemolytic action of staphylococcus toxin the same as red blood cells suspended in saline.

The failure of sulfapyridine to inhibit hemolysis is also shown by the following experiment: A group of blood agar plates, containing varying amounts of sulfapyridine, was prepared in the same manner as those previously described in this paper. Three drops of staphylococcus toxin were carefully dropped from a capillary pipette onto the surface of the plates. After two hours the toxin was absorbed, and the plates were placed in the incubator at 37.5° C. There was no difference in the size of the hemolytic zones produced by the toxin on the plates containing sulfapyridine and the control medium. This hemolysis is shown in Fig. 2. The blood agar in *A* contains 10 mg. per cubic centimeter of sulfapyridine, while *B* contains no sulfapyridine.

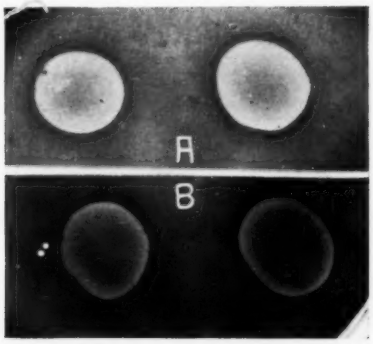


Fig. 2.—Hemolysis produced by staphylococcus toxin on blood agar plates. *A*, Plate contains 10 mg. of sulfapyridine per cubic centimeter of blood agar. *B*, Control for *A*. The degree of hemolysis is the same on both plates after twenty-four hours.

Fig. 3.—Skin necrosis produced by the intradermal injection of staphylococcus toxin in the rabbit. One-tenth cubic centimeter of toxin diluted 1:10 with saline was injected at *A*. A similar amount of toxin with 10 mg. sulfapyridine per cubic centimeter was injected at *B*. Photographed after twenty-four hours.

EFFECT OF SULFAPYRIDINE ON SKIN-NECROTIZING FACTOR IN STAPHYLOCOCCUS TOXIN

Four rabbits were used in this experiment. The skin was carefully shaven twenty-four hours or longer before the toxin was injected intradermally. Duplicate injections were made into each animal. The staphylococcus toxin was diluted in saline in which there was 10 mg. of sulfapyridine per cubic centimeter. Two rabbits were given 0.1 c.c., and two were given 0.2 c.c. of the toxin-sulfapyridine mixture. A similar concentration of toxin in saline was given each rabbit for the control. There was some variation in the size of the necrosis in the same rabbit where the toxin was injected and also where the toxin-sulfapyridine mixture was given. The necrosis was smaller in some of the areas where sulfapyridine and toxin were injected than it was where only the toxin was given. This variation is not significant, however, since there is a variation in the size of the skin necrosis when the same amount

of toxin is given to a single rabbit. Fig. 3 shows the necrosis produced by 0.1 c.c. of staphylococcus toxin given intradermally at *A*, and the necrosis produced by a similar amount of staphylococcus toxin and sulfapyridine at *B*.

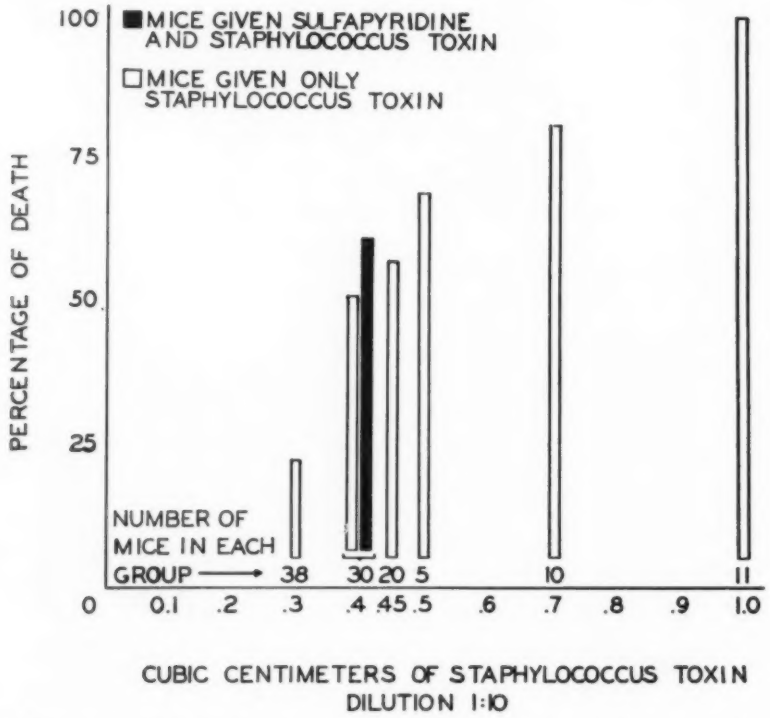


Chart 2.—Percentage of death in mice given staphylococcus toxin intraperitoneally and also staphylococcus toxin containing 20 mg. of sulfapyridine per cubic centimeter.

EFFECT OF SULFAPYRIDINE ON LETHAL FACTOR IN STAPHYLOCOCCUS TOXIN

Normal white mice were used in this study. The toxin was given intraperitoneally, and the animals were observed for forty-eight hours. The percentage of death was based upon the number of mice dying during this period. The toxin was diluted 1:10 with saline. Twenty milligrams of sulfapyridine per cubic centimeter were added to this diluted toxin. The toxin-sulfapyridine suspension and the toxin were injected immediately following dilution. The number of mice injected and the per cent of death are shown in Chart 2. It is evident from these data that sulfapyridine in the concentration used, when given at the same time as the toxin, has no inhibitory action on the lethal factor in this toxin.

DISCUSSION

It is evident from the data presented in this paper that sulfapyridine extract broth is bacteriostatic for staphylococci. The degree of inhibition in growth apparently is proportional to the concentration of the drug as used in these experiments. This inhibitory action of sulfapyridine on staphylococci apparently can be demonstrated more easily with a small inoculum.

Different strains of staphylococci vary in their susceptibility to the action of sulfapyridine. It is interesting to observe that the strain of staphylococcus most susceptible to the action of sulfapyridine in this experiment is a non-toxin-producing organism. In contrast to this, the two strains of staphylococci most resistant to this drug are both toxin-producing organisms. It will be necessary to make subsequent studies to determine whether there is any correlation between toxin production and the bacteriostatic resistance of staphylococci to sulfapyridine.

The failure of strain "7" to grow in sulfapyridine within a period of seventy-two hours suggests that this drug may be bactericidal. The absence of growth in extract broth in the tubes containing sulfapyridine suggests that certain strains of staphylococci were either killed by this chemical or died spontaneously during the interval.

The appearance of a granular type of growth in the tubes containing sulfapyridine after forty-eight to seventy-two hours might suggest that the bacteria were clumped as a result of the action of sulfapyridine. The diminution in growth of the staphylococci parallels the development of these granules. These observations indicate that there is a variation in the susceptibility of the three strains of staphylococci to a similar concentration of sulfapyridine. Keefer and Rantz[4] have also found a considerable variation in the bactericidal effect of sulfanilamide on different serologic types of streptococci. Meyer[5] has described a clumping of hemolytic streptococci in a serum medium containing a sulfanilamide-sugar compound, which is apparently similar to that observed in the present experiments with staphylococci grown in sulfapyridine.

All the experiments in which staphylococcus toxin and sulfapyridine were used do not give any indication that this drug inhibits any of the actions of this toxin. This observation is interesting in view of Carpenter's studies with staphylococcus toxin and sulfanilamide. Carpenter and his associates[6, 7] found that the lethal action of staphylococcus toxin for mice was inhibited when the toxin was mixed with sulfanilamide preceding an intraperitoneal injection. Rigdon and Avery, in unpublished experiments, found that the hemolytic and skin-necrotizing action of staphylococcus toxin was not inhibited by either sulfanilamide or sulfapyridine. The inhibition of hemolysis about colonies of staphylococci growing on blood agar plates in the presence of sulfapyridine is apparently due to the bacteriostatic action of the drug on the organism and not to any specific effect of the drug upon the hemolysin.

SUMMARY

Sulfapyridine in extract broth has a bacteriostatic effect upon staphylococci. There is, however, a considerable variation in the action of this drug on different strains of staphylococci. There is some indication from this study that sulfapyridine may be also bactericidal for some strains of staphylococci when grown in vitro.

The hemolytic, the skin-necrotizing, and the lethal factors in staphylococcus toxin apparently are not affected by sulfapyridine.

REFERENCES

1. Fenton, W. J., and Hodgkins, F.: Staphylococcal Septicemia, Treated by M. & B. 693, Lancet **2**: 667, 1938.
2. Whitby, L.: Chemotherapy of Bacterial Infections, Lancet **2**: 1094, 1938.
3. Long, P. H.: Sulfapyridine, J. A. M. A. **112**: 538, 1939.
4. Keefer, C. S., and Rantz, L. A.: Sulfanilamide. A Study of Its Mode of Action on Hemolytic Streptococci, Arch. Int. Med. **63**: 957, 1939.
5. Meyer, F.: New Studies in Sulfanilamide Therapy, Quart. Bull. Sea View Hosp. 3: 380, 1938.
6. Carpenter, C. M., Barbour, G. M., and Hawley, P. L.: The Inactivation of Staphylococcal Toxin in Vitro by Sulfanilamide, J. Pediat. **14**: 116, 1939.
7. Carpenter, C. M., and Barbour, G. M.: Inactivation of Toxins of Staphylococcus aureus and Clostridium welchii in Vitro by Sulfanilamide, Proc. Soc. Exper. Biol. & Med. **41**: 354, 1939.

THE ACTION OF DIGITALOID GLUCOSIDES ON THE VASOMOTOR CENTER*

PHILIP BLICKENSDORFER, PH.D., AND H. A. MCGUIGAN, M.D.
CHICAGO, ILL.

IN ANIMAL experiments, digitalis and its allies, when injected intravenously, may raise the blood pressure as high as 300 mm. Hg or more. The efficiency of the heart, as shown by Cushny,[1] is insufficient to account for the whole phenomenon. It is assumed that the vasomotor center is involved, and that the rise is due to a constrictor action on the arterioles. The object of this report is to present evidence of a direct action of digitaloid glucosides on the vasomotor center.

Digitalis Given Intravenously.—The average fatal dose of digitalis of the strength of the International Standard is 1.2 mg. per kg. weight in the dog, when given at the rate of 0.1 c.c. per kg. every five minutes.[2] The pressure may rise slightly from the beginning, but not significantly, until *about* one-half the toxic dose has been administered, that is, until toxic symptoms begin to be manifest. In most cases the total rise is about 60 mm. Hg. In a smaller percentage of cases the rise may be enormous. This rise, occurring only in the toxic stage, suggests a central action, since the heart at this time is becoming inefficient.

Although digitalis injected intravenously may cause at first a sharp, temporary fall of blood pressure, this does not occur with proper administration. A fall of pressure is due to too rapid injection, injection shock, which may be elicited by almost any substance injected rapidly. In the present work, we present evidence to show the direct action of digitalis on the centers in the nervous system.

Method.—Dogs are anesthetized with intraperitoneal injections of 35 mg. of pentobarbital per kilogram body weight. To show central action the dig-

*From the Department of Pharmacology, University of Illinois, College of Medicine, Chicago.

Received for publication, September 23, 1939.

italoid drug is injected directly into the fourth ventricle. A continuous record of the blood pressure before and after the injection is made.

Injection is easily done by inserting a hypodermic needle into the region, making the puncture about 1 inch to 1½ inches below the occipital protuberance, with the head bent sharply toward the chest. The flow of the clear fluid is proof of the location. Following such puncture, however, the blood pressure returns rather rapidly to the normal level (Fig. 1).

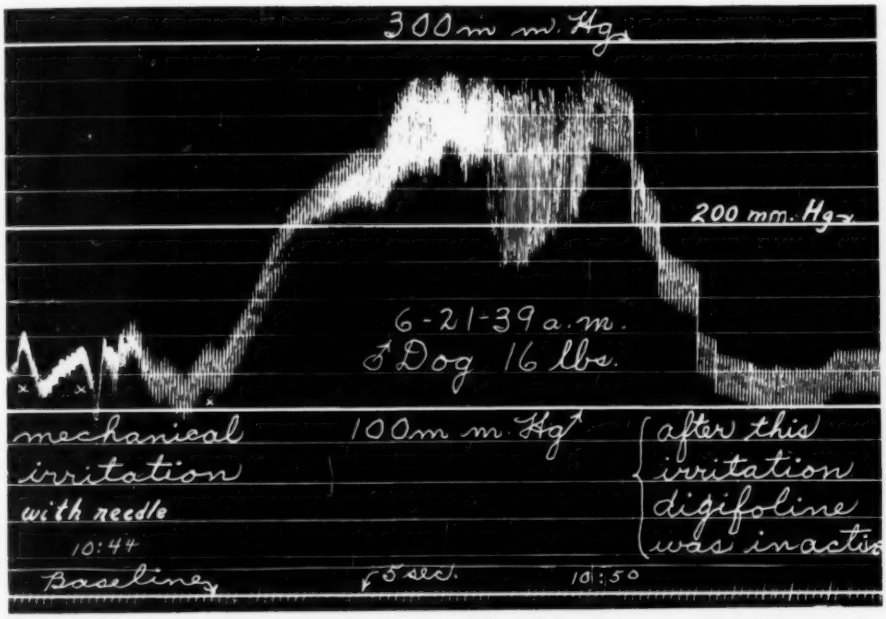


Fig. 1.—Rapid rise in blood pressure induced by mechanical irritation of floor of fourth ventricle by hypodermic needle.

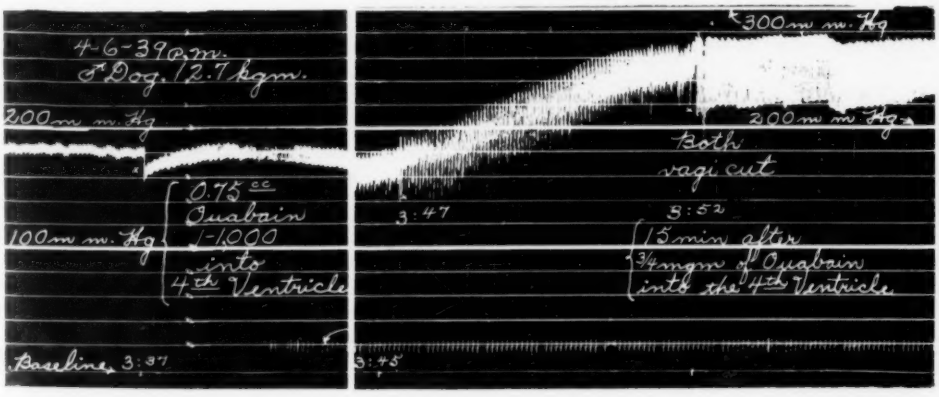


Fig. 2.—Rise in blood pressure effected by 0.75 mg. ouabain injected into the fourth ventricle.

One may occasionally irritate the vasomotor center or tract and immediately raise the pressure to enormous heights (300 mm. Hg). After such injuries digitalis may be inactive centrally. The rise due to digitaloid drugs has a slower onset and persists much longer.

Effect of Digitaloid Drugs Is Not Due to Irritant Action.—It is necessary to rule out local irritation. It is well known that an irritant action readily occurs if hypertonic salt solutions are injected into this region. We believe irritation by hypotonicity or hypertonicity has been ruled out by the slow effect in many cases, and also by the absence of irritative reactions, such as muscular twitching, or immediate change in blood pressure.

Action of Ouabain.—We commenced with ouabain because of the very dilute solutions needed, and because it furnished a clear solution comparable with the cerebrospinal fluid. Fig. 2 shows the typical effect of ouabain.

Digifoline N.N.R.—Sterilized digifoline contains no alcohol or glycerin, and is a clear solution containing the active ingredients of 0.1 Gm. digitalis leaf in 2 c.c. Fig. 3 shows the effect of this preparation.

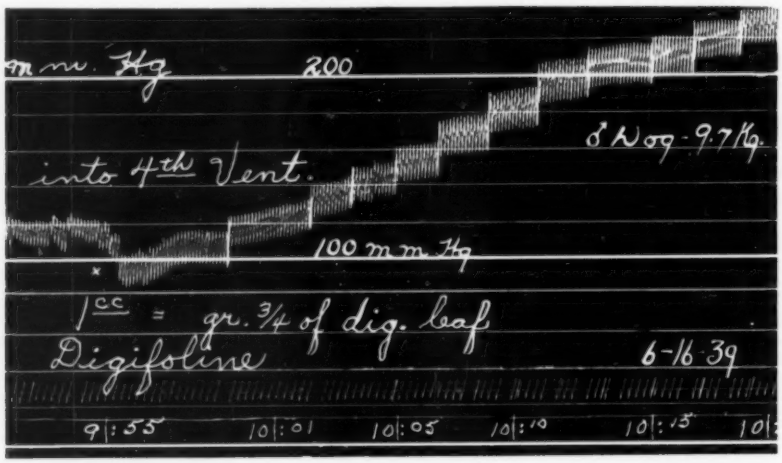


Fig. 3.—Change in blood pressure effected by digifoline injected into the fourth ventricle.

Fig. 4 shows the effect of digifoline evaporated and dissolved in 70 per cent alcohol. Note that the action develops rapidly and persists. Alcohol alone has no such action on blood pressure. It is rather surprising the volume of alcohol that may be injected without affecting the blood pressure or preventing the digitalis action.

That alcohol has little influence, except in hastening the onset of action, due presumably to hastening penetration, is shown by Fig. 5. We have obtained similar effects with an infusion of digitalis.

Paralysis of the Vasomotor Center by Alcohol.—One can, with very large doses of alcohol, paralyze the vasomotor center so that digitalis given centrally has no effect. Fig. 6 shows this effect. Note that in this experiment, when relatively large amounts of spinal fluid were replaced with alcohol, the tincture of digitalis centrally caused no rise in blood pressure, but the peripheral action of digitalis was still obtainable.

Relative Susceptibility of the Vital Centers to Alcohol.—In our experience in this work the respiratory center is the first to be paralyzed, so that artificial respiration is needed throughout the experiment. The vagus center, as in-

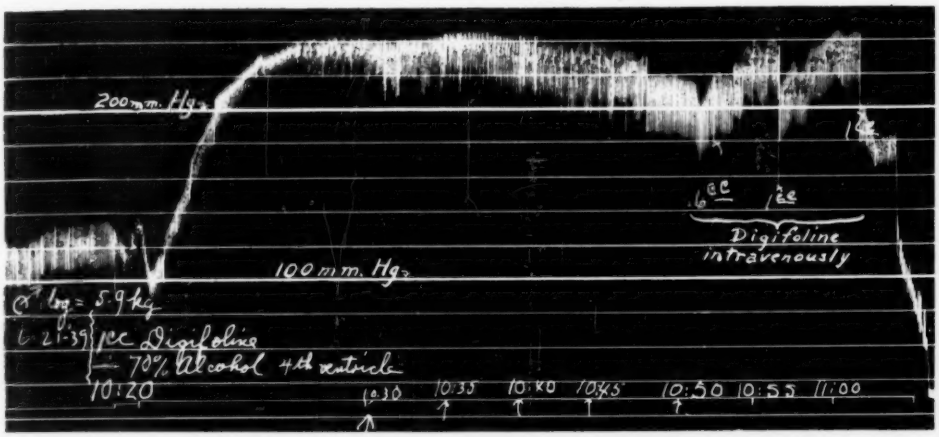
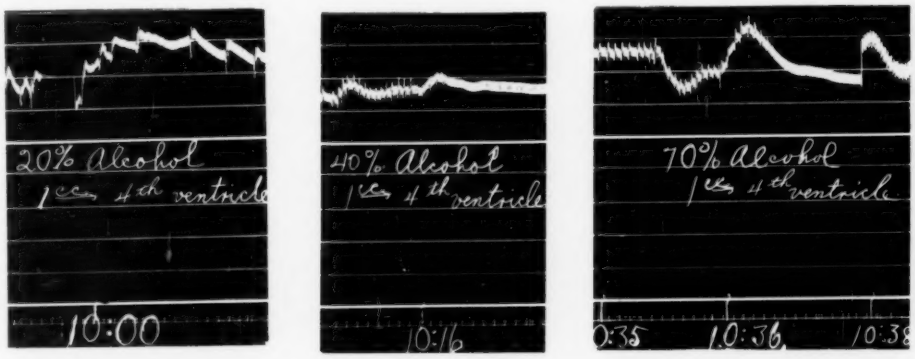


Fig. 4.—Rapid rise in blood pressure effected by digifoline residue dissolved in 70 per cent alcohol. Compare with more gradual rise shown in Fig. 3.



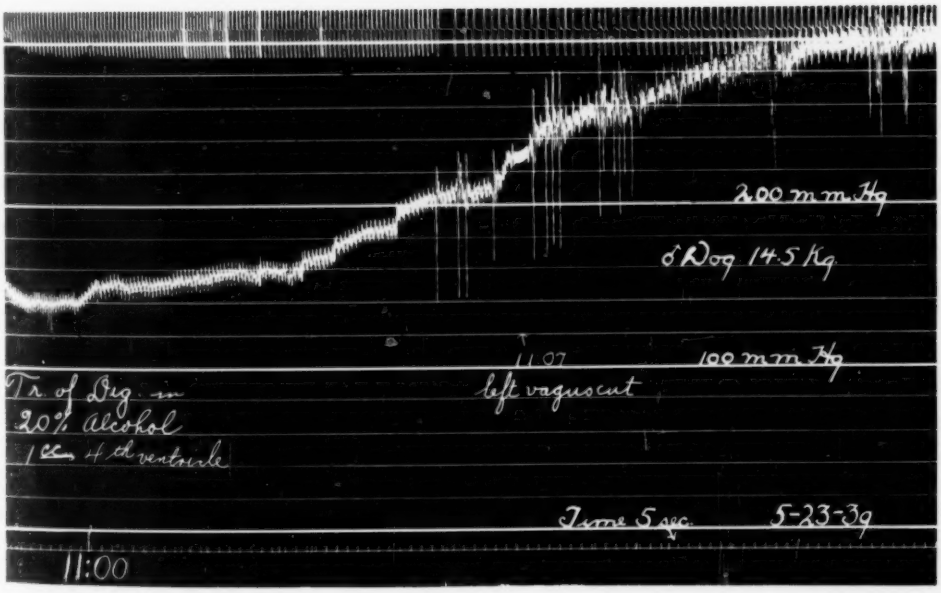


Fig. 5.—Small quantities of alcohol injected into the fourth ventricle have no appreciable effect on the blood pressure, while tincture of digitalis residue suspended in 20 per cent alcohol effects a rise in blood pressure to 310 mm. Hg.

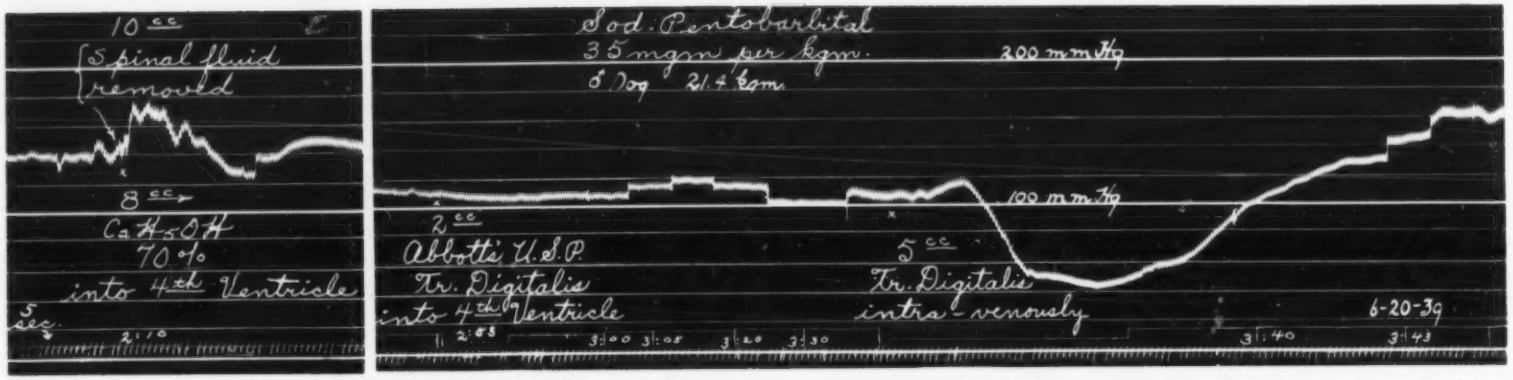


Fig. 6.—A large quantity of 70 per cent alcohol paralyzes the vasomotor center. Tincture of digitalis placed into the fourth ventricle did not effect a rise in blood pressure, but when given intravenously caused an appreciable rise in blood pressure.

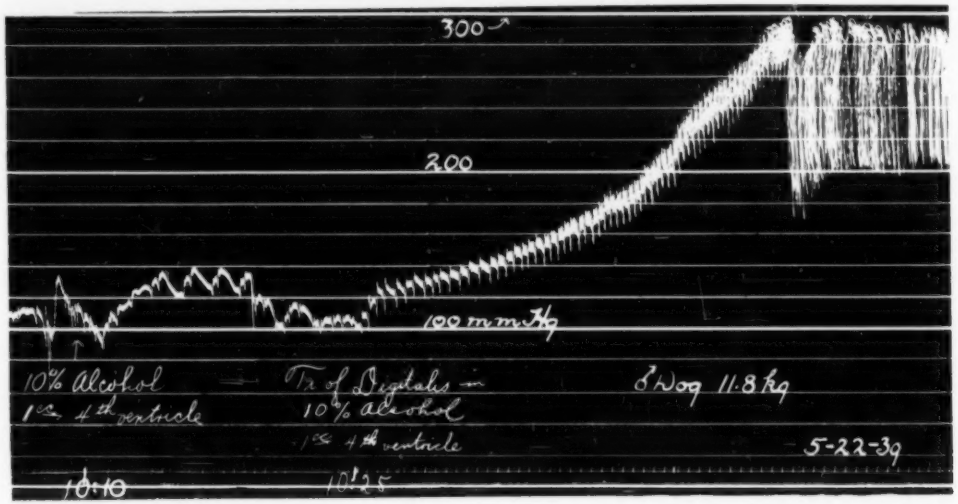


Fig. 7.—While 10 per cent alcohol injected into the fouth ventricle effects but a very slight rise in blood pressure, tincture of digitalis residue suspended in 10 per cent alcohol effects a very rapid marked rise in blood pressure.

dicated by the effect on the blood pressure tracings, is the second to be paralyzed, while the vasomotor center is much more resistant.

Tincture of Digitalis.—A commercial standard tincture (containing 70 per cent alcohol), which we had tested in the usual way on dogs, was given intravenously. It was necessary first to test the effect of the alcohol. If properly injected, alcohol exerts a depressant action only. Occasionally, however, one may get a pronounced rise of pressure. Indeed one may get a rise to 300 mm. Hg in this way, which is not due to alcohol but to direct stimulation with the needle. It is necessary, therefore, to rule out puncture stimulation. One may inject several times the volume of alcohol (3 c.c.) used in the tincture of digitalis (1 c.c.) without preventing the digitalis rise in pressure. Such doses of alcohol usually paralyze the respiratory center so that artificial respiration is necessary. By injecting larger volumes of alcohol, one may depress the center to such a degree that digitalis following the alcohol is inert. Fig. 7 shows the typical effect of a solution of digitalis in dilute alcohol injected into the fourth ventricle.

SUMMARY AND CONCLUSIONS

The central action of digitaloid glucosides in raising the blood pressure is shown. That there is also a peripheral factor involved is also indicated. After the central action has reached its maximum in many cases an added rise may be obtained by injecting the drug intravenously.

REFERENCES

1. Cushny, A. R.: Digitalis and Its Allies, New York, 1925, Longmans, Green & Co., p. 145.
2. McGuigan, R. A., and McGuigan, H. A.: J. Pharmacol & Exper. Therap. **63**: 76, 1938.

QUANTITATIVE PROTHROMBIN AND HIPPURIC-ACID DETERMINATIONS AS SENSITIVE REFLECTORS OF LIVER DAMAGE IN HUMAN SUBJECTS*

SLOAN J. WILSON, M.D., COLUMBUS, OHIO

WITH the development of quantitative methods for the determination of plasma prothrombin by Quick,[1] and Warner, Brinkhous, and Smith,[2, 3] the liver was established as an extremely important intermediary in the production of this coagulation element. Various investigators have reported a decrease in prothrombin associated with liver damage, but no attempt has been made to correlate the level of the plasma prothrombin with varying degrees of hepatic damage or with the results of the various common liver function tests.[4] Smith, Warner, and Brinkhous reported a marked decrease in prothrombin after severe chloroform-induced liver damage in dogs. The extirpation of 60 to 75 per cent of the liver in rats[5] resulted in a temporary decrease of 30 to

*From the Division of Medical Research, Department of Medicine, Ohio State University, Columbus.

Aided by a grant from the Comly Research Fund, Ohio State University.
Received for publication, September 27, 1939.

40 per cent in plasma prothrombin, with a subsequent return to normal during the ten days to three weeks required for the regeneration of the liver to its normal weight. Scanlon, Brinkhous, Warner, Smith, and Flynn[6] failed to produce an increase in plasma prothrombin with bile salts and vitamin K in an individual with cirrhosis of the liver. Stewart[7] noted that when hypoprothrombinemia occurred with obstructive jaundice, the more severe the liver damage the less marked was the prothrombin recovery with therapy consisting of bile salts and vitamin K.

It has been shown that there is no apparent correlation or relationship between fibrinogen and prothrombin.[3, 7] In the present study, observations have been made on normal human subjects and on patients with various degrees of liver damage without biliary obstruction or fistulas. The two-step method of Warner, Brinkhous, and Smith was used to determine the level of the plasma prothrombin. The prothrombin, expressed in per cent of normal, was then compared with the plasma fibrinogen and the galactose, bromsulphalein, and Quick hippuric acid function tests.[8]

The one-step methods of Quick,[1] and Smith, Ziffren, Owen, and Hoffman[9] do not measure the prothrombin quantitatively in every instance[10]; therefore they were not used in this study. This is particularly true in some patients with hypoprothrombinemia, who have developed a rapid conversion rate of prothrombin to thrombin as a compensatory mechanism. In such instances, a prothrombin level of 30 to 40 per cent of normal, as determined by the two-stage method, may give a reading of 80 to 100 per cent of normal by methods which measure in one step the rate of conversion of prothrombin to thrombin and the interaction of thrombin and fibrinogen to form the fibrin clot.

OBSERVATIONS

In a series of 41 patients without obstructive jaundice or biliary fistulas the quantitative level of the plasma prothrombin was found to correlate closely with the quantity of hippuric acid excreted (Fig. 1). In addition to normal individuals the series included persons with cirrhosis of the liver, Banti's syndrome, familial hemolytic icterus, pernicious anemia, aplastic anemia, multiple myeloma, polycythemia vera, Hodgkin's disease, and leucemia of all types with various degrees of liver infiltration. In those individuals with proved liver damage in which the hippuric acid excretion was 0.86 to 2.0 Gm., the prothrombin was found to be 19 to 37 per cent of normal; with 2.0 to 3.9 Gm. of hippuric acid excretion, the prothrombin level was 33 to 90 per cent of normal; and with 3.9 to 4.56 Gm. hippuric acid excretion, the prothrombin level was 70 to 100 per cent of normal.

There was no consistent correlation between plasma prothrombin and plasma fibrinogen, particularly when an infectious process was present. In one patient with aplastic anemia, oral sepsis, continuous high fever of 104° F., and a resulting acute hepatic damage, who was given daily transfusions, the prothrombin was only 30 per cent of normal, but the fibrinogen was elevated to 0.531 Gm. per cent. The icterus index was 24 units. On admission the prothrombin was 94 per cent of normal, and the fibrinogen was 0.483 Gm. per cent. In another individual with aplastic anemia the admission prothrombin

was 69 per cent of normal, the fibrinogen 0.321 Gm. per cent, and the icterus index 6 units. This patient subsequently developed an infection in the gluteal region, sustained a high elevation in temperature, became jaundiced (icterus index 78), the fibrinogen became elevated to 0.518 Gm. per cent, but the prothrombin decreased to 24 per cent of normal.

The galactose tolerance test was within normal limits in all patients studied. There was a wide variation in the results recorded for the bromsulphalein dye test.

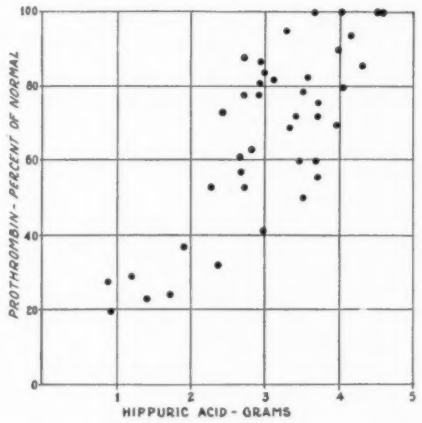


Fig. 1.—Illustrating the close correlation of plasma prothrombin determination (ordinate) and the Quick hippuric acid test (abscissa) in terms of liver damage as measured by these respective tests. Any decrease in the hippuric acid output was always accompanied by a proportionately diminished prothrombin level in the blood. Each point represents a different patient with a total of 41.

The following two cases of advanced hepatic cirrhosis illustrate the variation in the different liver function tests and the levels of plasma prothrombin and fibrinogen.

CASE 1 (Fig. 2).—R. W., a white male, aged 13 years, was admitted to the University Hospital with the complaint of frequent attacks of epistaxis and bleeding gums of about three years' duration. Positive physical findings on admission included icteric sclera, hemorrhagic gums, coagulated blood in the nares, and enlarged liver and spleen. A diagnosis was made of hepatic cirrhosis with splenomegaly (Banti's syndrome). The plasma prothrombin was 24 per cent of normal, and the fibrinogen was 0.288 Gm. per cent. Liver function tests: Quick hippuric acid test 1.71 Gm.; bromsulphalein dye retention test (5 mg. per kg. body weight) 10 per cent in one-half hour and 5 per cent in one hour; galactose tolerance test 2.6 Gm. The icterus index was 13.3 units. After a month's observation the patient was given intensive therapy with bile salts and vitamin K (the extract from 200 Gm. of alfalfa daily for a period of three months). There was only a negligible variation in the level of the plasma prothrombin. The patient continued to have occasional attacks of epistaxis and bleeding gums but gained in weight and carried on the normal activities of a boy his age.

CASE 2 (Fig. 3).—J. K., a white female, aged 15 years, vomited a large quantity of blood, necessitating two emergency blood transfusions, the day before admission to University Hospital. She had had previous attacks of what was thought to be catarrhal jaundice, for which sulfanilamide therapy had been prescribed. On physical examination there was a slight icteric color to the sclera and the skin. The liver was tender to palpation. The spleen later became palpable and ascites developed. The prothrombin on admission, in spite of 1,000 c.c. of blood received by transfusion, was only 28 per cent of normal, and the fibrinogen was 0.207 Gm. per cent. The icterus index was 25 units. Liver function tests: Quick hippuric

acid test 1.4 Gm.; galactose tolerance test 3.35 Gm. Bile salts and vitamin K (the extract of 400 Gm. of alfalfa) were given daily for ten days, with no appreciable change in the level of the plasma prothrombin. During an attack of acute parotitis with an elevation in temperature, there was an increase in the fibrinogen with no variation in the prothrombin level. She died following a severe hematemesis. At autopsy the diagnosis was made of toxic cirrhosis with an early attempt at liver cell regeneration, ascites, splenomegaly, and bilateral hydrothorax.

DISCUSSION

There is a definite correlation between the level of the plasma prothrombin and the synthesis and excretion of hippuric acid following the ingestion of sodium benzoate, as measured by the method of Quick. Observations confirmed at operation and autopsy have shown that these two tests are sensitive reflectors of liver damage. Other investigators have reported that the hippuric acid test is a good index of the amount of hepatic damage present.[11-13] The observations of Warner[5] with partial extirpation of the liver in rats and the resulting hypoprothrombinemia show that there is a definite relationship between the amount of liver damage and the level of the plasma prothrombin. Vitamin K and bile salts have no effect on the level of the prothrombin when the hypoprothrombinemia is due to extensive injury to the parenchymatous tissue of the liver. A decrease in prothrombin in patients without obstructive jaundice or biliary fistulas may not be due to lack of vitamin K or to any decrease in the absorption from the intestinal tract.[6] In our experience individuals with mild chronic pathologic states frequently show a decrease of 10 to 25 per cent in the level of the plasma prothrombin.

The methods most widely used at the present time for the quantitative determination of prothrombin are the two-stage titration technique of Warner, Brinkhous, and Smith,[2, 3] and the one-stage techniques of Quick,[1] and Smith, Ziffren, Owen, and Hoffman.[9] The two-stage titration technique is undoubtedly the more accurate but it is difficult to use as a routine laboratory procedure. In many instances, however, the one-step method does not measure the quantity of prothrombin because it is an index of many variable factors, including (1) the amount of prothrombin, (2) the rate of conversion of prothrombin to thrombin, and (3) the reaction of thrombin with fibrinogen to form the fibrin clot. In the two-step method, the prothrombin is first converted to thrombin, and the thrombin is then titrated. There is a wide variation in the conversion rate in various species.[14] The ease with which this factor may vary and the differences in the results of the two methods have been conclusively demonstrated in infants during the first few days of life, in which the prothrombin may actually be very low, but because of the rapid conversion rate normal values are recorded by the one-step methods.[10] In one of our patients (Case 1, R. W.) in whom there was a consistent hypoprothrombinemia secondary to cirrhosis of the liver, the tendency to hemorrhage was variable. At times there was a definite compensatory mechanism in which the conversion rate was very rapid, the actual prothrombin value being 32 per cent of normal as measured by the two-step method, and 80 per cent of normal by the one-step method. Inasmuch as the one-step technique in some instances does not accurately determine the amount of prothrombin,

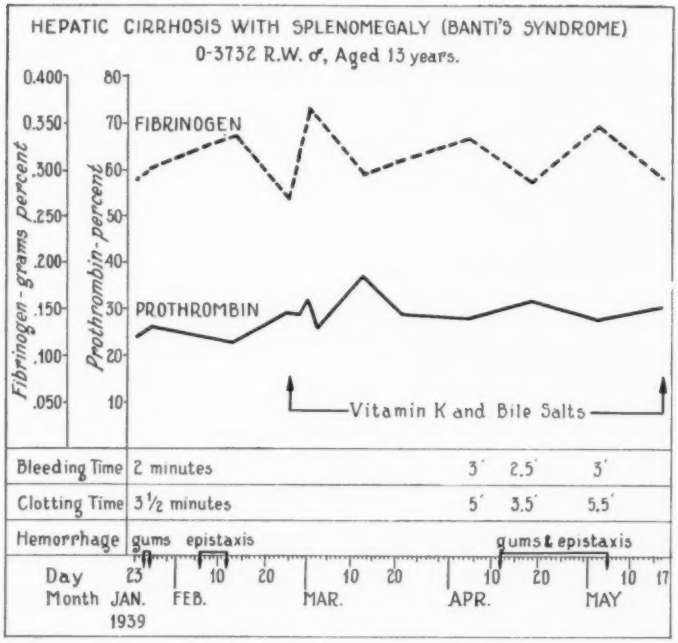


Fig. 2.—Illustrating the lack of response of the plasma prothrombin to seventy-nine days' intensive therapy with vitamin K and bile salts in an individual with advanced hepatic cirrhosis and splenomegaly (Banti's syndrome). The fibrinogen remained consistently within normal limits. The Quick hippuric acid test was 1.71 Gm.; galactose tolerance test 2.6 Gm.; and the bromsulphalein dye retention test 10 per cent in one-half hour, 5 per cent in one hour.

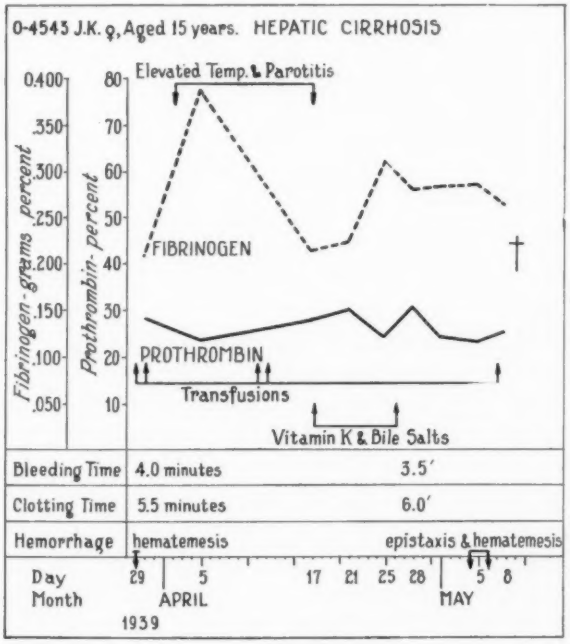


Fig. 3.—Illustrating the lack of response of the plasma prothrombin to ten days' intensive therapy with vitamin K and bile salts in an individual with toxic hepatic cirrhosis. There was a rise in the plasma fibrinogen during an attack of acute parotitis with an elevation in temperature. Transfusions had very slight effect on the level of the plasma prothrombin.

being nevertheless an excellent practical measure of the tendency to hemorrhage, the results of such tests cannot be used as indices of liver function.

There is no consistent correlation between the levels of the prothrombin and fibrinogen in the plasma. Smith, Warner, and Brinkhous[3] reported that the prothrombin formation is more easily disturbed than fibrinogen formation, and observed an increase in fibrinogen with a sustained low prothrombin level during an episode of canine distemper which complicated the recovery of one of their dogs following chloroform-induced hepatic damage. In the two patients already cited, who developed a sustained high temperature secondary to infection and an acute toxic hepatitis, the same phenomenon was observed. It has been shown that uncomplicated fever induced by the Kettering hypertherm may produce liver damage with a resulting hypoprothrombinemia but that the fibrinogen may not be affected.[15] Ham and Curtis[16] observed only slight variations in the fibrinogen level when hyperpyrexia was induced by high environmental temperature, whereas following intravenous injections of typhoid vaccine, the level rose sharply. The level of the plasma prothrombin may be decreased if there is liver damage due to any cause, but there is no stimulation to an increased overproduction with concurrent infections. Stewart[7] emphasized the lack of correlation between the quantitative prothrombin and the fibrinogen determinations. It would seem that the level of the fibrinogen is an extremely poor index of the relative degree of hepatic damage.

A relatively extensive degree of damage to the parenchymatous tissue of the lever must be present before it is reflected in many of the commonly used liver function tests. This is easily understood as Mann[17] has shown that many of the physiologic functions of the liver may be maintained at normal levels with only a very small portion of the liver intact, or after generalized damage to the entire parenchymatous tissue. Apparently the ability of the liver to form prothrombin from vitamin K and to synthesize hippuric acid from sodium benzoate is a more sensitixe measure of the amount of liver damage present than the majority of the liver function tests.

It is interesting to note that prothrombin cannot be replaced by blood transfusion in sufficient quantity to be of any therapeutic value. The changes in the total content of prothrombin in the plasma of the recipient are dependent on the prothrombin content of the plasma of the donor, and may be calculated on the basis of the direct additive[18] effect. Theoretically, if a transfusion of 500 c.c. of blood containing the normal quantity of prothrombin is given to a person weighing 70 kg., the level of the plasma prothrombin would be elevated only 7 per cent. In one of the cases of aplastic anemia with a prothrombin of 30 per cent of normal, daily transfusions were given without controlling the hemorrhagic diathesis produced by a combined hypoprothrombinemia and thrombocytopenia. In another patient with a pretransfusion level of 23 per cent of normal, the prothrombin was 28 per cent of normal after two transfusions of 500 c.c. each. Stewart[7] observed that blood transfusions appeared to be a rather inefficient method of combating the bleeding tendency due to hypoprothrombinemia, as the effect on the recipient's

plasma prothrombin is slight and transitory, and reported an increase of only 6 per cent in one adult patient whose plasma prothrombin was determined before and after transfusion of 600 c.c. of blood.

CONCLUSIONS

1. In the patients here studied, without biliary obstruction or biliary fistulas, the quantitative levels of plasma prothrombin and the amounts of hippuric acid excreted following the ingestion of a known quantity of sodium benzoate (Quick test) would seem to have reflected most sensitively and consistently the existing degree of liver damage. There was no such suggestive correlation observed between these tests and the plasma fibrinogen levels, the bromsulphalein dye clearance or galactose utilization, either singly or collectively, when all were studied in the same patient.

2. The one-step methods of Quick, and Smith, Ziffren, Owen, and Hoffman do not accurately determine the true quantity of prothrombin in the blood plasma of some individuals because of the variability in the rate of its conversion to thrombin; hence in some instances the results of the one-step techniques, though they are an index of the tendency to bleed, cannot be correlated with variations in liver function.

3. Transfusions are of little or no therapeutic value in the control of the hemorrhagic tendency in individuals with hypoprothrombinemia.

REFERENCES

1. Quick, A. J., Stanley-Brown, M., and Bancroft, F. W.: Am. J. M. Sc. **190**: 501, 1935; Quick, A. J.: Am. J. Physiol. **114**: 282, 1936; J. A. M. A. **110**; 1658, 1938.
2. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Am. J. Physiol. **114**: 667, 1936.
3. Smith, H. P., Warner, E. D., and Brinkhous, K. M.: J. Exper. Med. **66**: 801, 1937.
4. Wilson, S. J.: Proc. Soc. Exper. Biol. & Med. **41**: 559, 1939.
5. Warner, E. D.: J. Exper. Med. **68**: 831, 1938.
6. Scanlon, G. H., Brinkhous, K. M., Warner, E. D., Smith, H. P., and Flynn, J. E.: J. A. M. A. **112**: 1898, 1939.
7. Stewart, J. D.: Ann. Surg. **109**: 588, 1939.
8. Quick, A. J.: Proc. Soc. Exper. Biol. & Med. **29**: 12, 1932; Am. J. M. Sc. **185**: 630, 1933.
9. Smith, H. P., Ziffren, S. E., Owen, C. A., and Hoffman, G. R.: J. A. M. A. **113**: 380, 1939; Proc. Soc. Exper. Biol. & Med. **40**: 595, 1939.
10. Owen, C. A., Hoffman, G. R., Ziffren, S. E., and Smith, H. P.: Proc. Soc. Exper. Biol. & Med. **41**: 181, 1939; Ibid. **40**: 599, 1939.
11. Snell, A. M., and Plunkett, J. E.: Am. J. Digest. Dis. & Nutrition **2**: 716, 1936.
12. Kohlstaedt, K. G., and Helmer, O. M.: Am. J. Digest. Dis. & Nutrition **3**: 459, 1936.
13. Boyce, F. F., and McFetridge, E. M.: Arch. Surg. **37**: 401, 1938.
14. Warner, E. D., Brinkhous, K. M., and Smith, H. P.: Proc. Soc. Exper. Biol. & Med. **40**: 197, 1939; Ann. Int. Med. **13**: 1214, 1940.
15. Wilson, S. J., and Doan, C. A.: J. A. M. A. **112**: 877, 1939; Proc. Soc. Exper. Biol. & Med. **41**: 115, 1939.
16. Ham, T. H., and Curtis, F. C.: Medicine **17**: 413, 1938.
17. Mann, F. C.: Ann. Int. Med. **8**: 432, 1934.
18. Lord, J. W., Andrus, W. D., and Moore, R. A.: Proc. Soc. Exper. Biol. & Med. **41**: 98, 1939.

A REVIEW OF ADVANCES IN THE STUDY OF AURICULAR DISORDERS*

ALDO LUISADA, M.D., BOSTON, MASS.

CLINICAL research on disorders of the auricles contribute to our knowledge of the physiology and pathology of the heart. In man there is need to study the possibility of physiologic pathways through the auricular myocardium, the constant presence of which Eyster and Meek, Rothberger and his associates, and Condorelli have demonstrated in laboratory animals by means of ligatures.

Some pathways lead the stimulus directly from the sinoauricular node to the right auricle (superior sinoauricular bundle, inferior sinoauricular bundle, and anterior sinoauricular fibers). An important pathway (the Bachmann or interauricular bundle), among others, carries the stimulus from the sinoauricular node to the left auricle. Three principal pathways, as Condorelli has shown, carry the stimulus from the sinoauricular node to the auriculoventricular node: (1) the inferior sinoauricular nodal, or Wenckebach bundle, arises in the tail of the sinoauricular node and passes along the torus lowerii between the venae cavae to the interauricular septum; (2) the superior sinoauricular septal, or Condorelli bundle, accompanies for some distance the Bachmann bundle, then turns downward, penetrating the septum; (3) some fibers arise in the posterior part of the sinoauricular node, go around the superior vena cava, and then penetrate the septum. Before entering the auriculoventricular node, these three principal pathways meet at the Condorelli crossing point.

These pathways are not anatomically differentiated bundles in the sense of the bundle of His. Some represent only the shortest route, and, therefore, the pathway of impulse conduction; others seem to be functionally differentiated. When some of these pathways are mechanically interrupted, the stimulus follows a longer course, and changes in the shape of the electrical wave occur. The Condorelli crossing point, however, is exceptional in that ligation here produces complete auriculoventricular dissociation. Although the three classical electrocardiographic leads have thrown much light on auricular disorders, special leads have been devised for attempting to record separately and simultaneously the electrical waves in each auricle, trying to secure more exact and important information.

METHODS

In 1910 Lewis[1] observed that placing the electrodes on the chest wall in the vicinity of the right auricle caused the small and frequent oscillations given by patients with auricular fibrillation to be much higher and more distinct.

*From the Massachusetts General Hospital, Boston.
Received for publication, September 16, 1939.

In 1924 Ackermann[2] began a new search for such special leads, describing a method by which two needles were inserted under the skin in the second and fifth interspaces near the right sternal border. He observed that P waves taken in this way were particularly high.

Condorelli[3] in 1931 very carefully studied a patient with disordered auricular action, using a slight modification of Ackermann's method; the auricular waves, produced, he thought, by the right auricle (but actually in all probability by both auricles), were best recorded with the needles in the second and fourth interspaces. A new lead, with one needle in the third interspace at the left sternal margin and the other near the fifth dorsal vertebra on the left paravertebral line, gave a record of auricular waves which he thought might represent left auricular activity. Laufer,[4, 5] working in my laboratory, applied Condorelli's leads to further patients, and Lian, Merklen, and Odinet[6] described an anterior lead for the right auricle and a posterior lead for the left auricle.

Lieberson and Lieberson[7] in 1934 introduced their technique, which was an important advance, with one electrode in the esophagus and the other (the so-called "indifferent electrode") on the left leg.

In 1935 I[8] made a further advance in developing this new method of auricular registration: a thin bipolar sound placed in the esophagus near the left auricle recorded the waves of this auricle; another record of auricular action was made by means of two plates, one applied to the tongue and the other over the xiphoid process, to represent prevalently right auricular action.

After careful study of fourteen different combinations of leads on the same patient, the esophageal method was chosen because its aim of securing a differentiation from the right or combined auricular P wave (P right) of the left (P left) seemed a possibility for the following reasons:

1. Higher voltage of the left auricular wave as compared with the voltage of the ventricular complexes.
2. Shorter duration of the left auricular wave.
3. Shorter P-R interval.

The longer P-R interval and the higher voltage of the right auricular wave were the reasons that the mouth-xiphoid method was chosen. By this technique the registration of waves from what was thought to be the right auricle appeared as good as in Condorelli's method, while the left auricular waves were more satisfactory than in previous methods.

Laufer and Rubino[9-11] and Sossai[12] applied this technique systematically to all patients with cardiac disturbances in my laboratory for several years. Later, Brown,[13] Zàrday,[14] and recently Deglaude and Laubry,[15] and Spühler[16] applied the unipolar esophageal method to many patients with auricular disorders.

In 1937 de Chatel and Hussey[17] in Rothberger's laboratory recognized the possible clinical value of the bipolar esophageal method after applying it to the dog, but were uncertain of its preference to the unipolar method.

Sossai[12] made this clear, however, the following year from a comparative study of the two techniques on many patients with differing rhythms in the two auricles or with delayed conduction to the left auricle, disorders which will be explained more fully later.

With one electrode on a distant part of the body, the recorded P waves represent the activity in both auricles, even though the other electrode is placed near the left auricle. To record the activity in the two auricles separately, therefore, the so-called "indifferent electrode" cannot be used.* This is in accord with the recent conclusion of Kossmann and Rader.[19]

In man, records of activity in the proximal part of the left auricle by the bipolar esophageal method are usually excellent, and, as a rule, show no clear evidence of the more distant parts, including right auricular activity. When left ventricular hypertrophy is present, as in aortic regurgitation, small ventricular waves may appear. Registration of right auricular activity is less satisfactory because high ventricular waves are seen, showing that the currents of all the heart muscle are recorded; when there is marked delay in contraction of the left auricle, small inverted deflections mark the activity of the left auricle; they are, however, smaller than right or combined auricular waves.

The best comparative method is obtained by simultaneous records through the use of a double-string galvanometer. When this is impossible, it is necessary to take a mechanical record (e.g., a carotid pulse) with each electrocardiogram and to measure the interval from the beginning of the P waves to the beginning of the pulse wave, because in the esophageal lead the R wave is usually small and obscure. The interval between "P *right*" wave and "P *left*" wave can easily be measured on the records taken by means of the double-string galvanometer. Working with a single-string galvanometer and a mechanical record, this interauricular interval equals ("P *right*" wave → pulse) – ("P *left*" wave → pulse). In the new leads usually the shorter P-R interval is in the esophageal lead, because the excitation of the left auricle physiologically occurs slightly later than that of the right.

The proximity of the electrodes to the auricular musculature makes it almost certain that normally the entire electrical activity is recorded and that no isoelectric phase exists. When the left auricle is much enlarged, however, it is theoretically possible that the distance of the electrodes from parts of the auricle and the changing axis of the auricle relative to the electrodes produce an initial isoelectric phase of the "left P" wave. This would lessen the significance of a recorded delay in contraction of the left auricle.

The normal interval between the beginning of the "P *right*" wave and the beginning of the "P *left*" wave is so brief (0.01 to 0.02 second) that the various methods for registering auricular activity must be evaluated on subjects with abnormally long intervals or auricular dissociation.

*In experiments on the sinoauricular electrogram I[18] found that when one needle is placed in the sinus region and the other on the right foreleg, it is impossible to obtain a wave representing the sinus activity that is as short and early as that obtained by means of two needles placed in the sinus region.

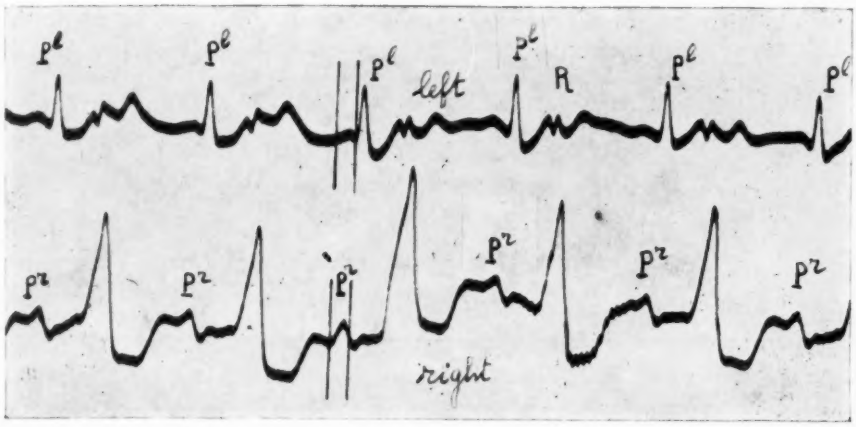


Fig. 1.—Delay of contraction of the left auricle in a patient with aortic regurgitation and coronary heart disease. Upper curve, esophageal lead for the left auricle. Lower curve, lead for the right auricle. (From Luisada.[5])

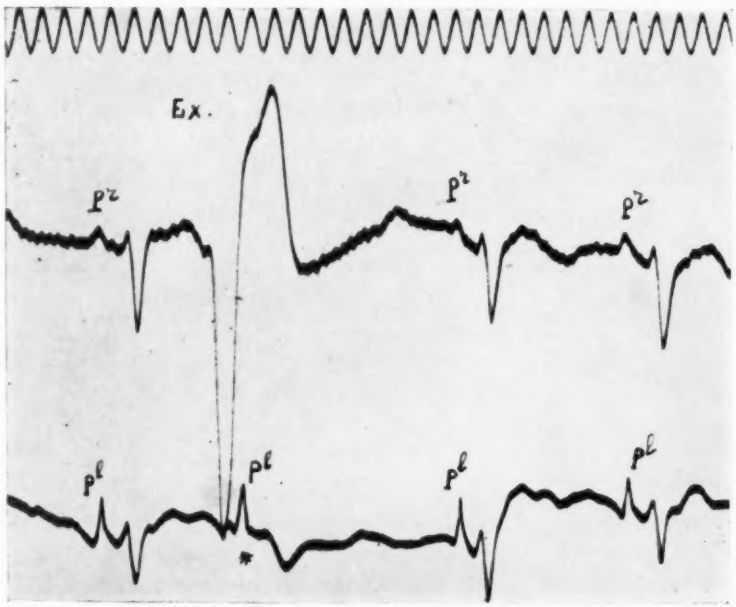


Fig. 2.—Ventricular premature beat. The esophageal lead permits the recording of the P left wave even during the premature beat (lower curve). (From Luisada.[5])

Comparing these new leads with the three classical leads, Laufer[4] found that the P wave is often wider in the latter. He found that the longer P-R interval recorded in the peripheral leads (that of the second lead) usually is equal to the P-R interval in the new lead for the right auricle. Differences in the P-R interval in the three classical leads, then, are probably due to the better or worse recording of the currents given from the two auricles in the different leads. Normally the P waves of the two auricles are not absolutely simultaneous, and in pathologic conditions they may be further separated.

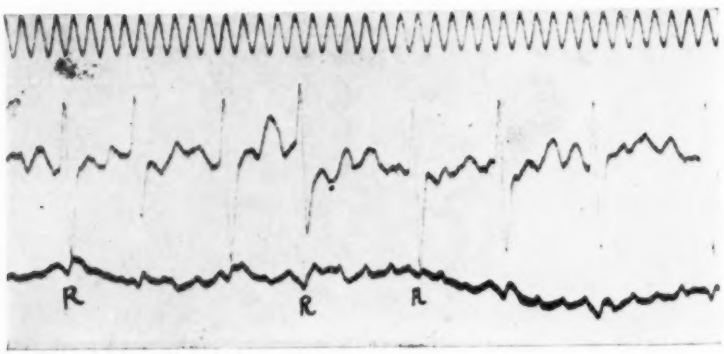


Fig. 3.—Flutter-fibrillation of both auricles. Upper curve, lead for the right auricle. Lower curve, esophageal lead for the left auricle. According to the circus movement hypothesis, the waves could be coarser in the right auricular curve because they are nearer to the circus. (From Rubino.[19])

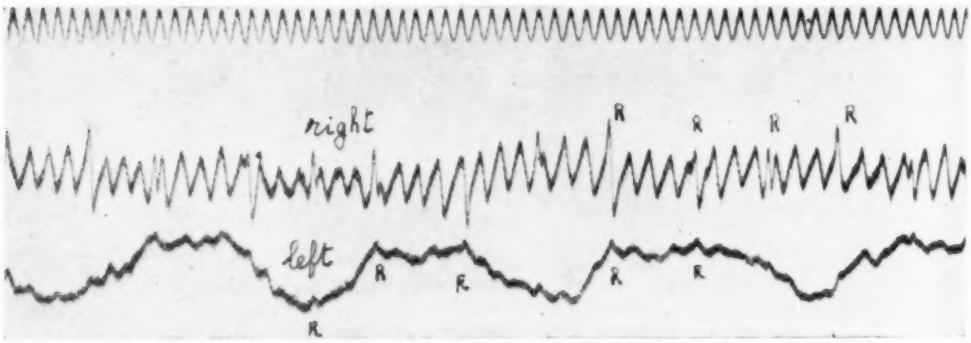


Fig. 4.—Flutter of the right auricle (upper curve), very small, irregular waves (?? fibrillation) in the left auricle (lower curve). It is possible that there is only one circus movement in the right auricle, the deflections in the left auricular curve being due to irregular radiations (From Rubino[16].)

CLINICAL INVESTIGATION OF AURICULAR DISORDERS

Scherf and Shookoff, Rothberger and Scherf, Condorelli, Scherf, and Siedeck, and others have studied intensively experimental disorders of the auricles; Groedel, Dressler, Mahaim, Condorelli, and many more have investigated clinical auricular disorders by means of the classical electrocardiographic leads. The new techniques already described have given a new stimulus for the study of the problems of the following possible auricular disorders:

1. *Delay in contraction of the left auricle.* Condorelli,[3] Laufer and Rubino,[9] Sossai,[12] and Luisada[19] have repeatedly observed a delay of the left auricular contraction in hearts with enlargement of the left auricle in mitral disease, or with myocardial fibrosis due to coronary atherosclerosis or syphilitic atresia of the coronary mouths. The delayed P wave arising from the left auricle may be either of normal height and form, or very small—as Rubino[10] and Sossai[12] have each described in a patient just after the change from auricular fibrillation to sinus rhythm. In cases with prolonged conduction from the sinoauricular node to the left auricle, the classical leads show large, double, or diphasic P waves.

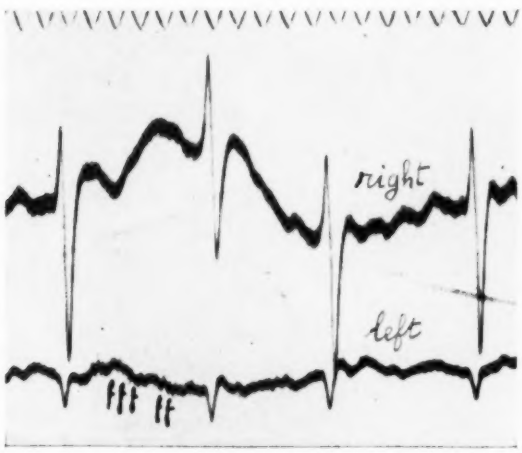


Fig. 5.—Fibrillation of both auricles. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. (From Luisada.[20])

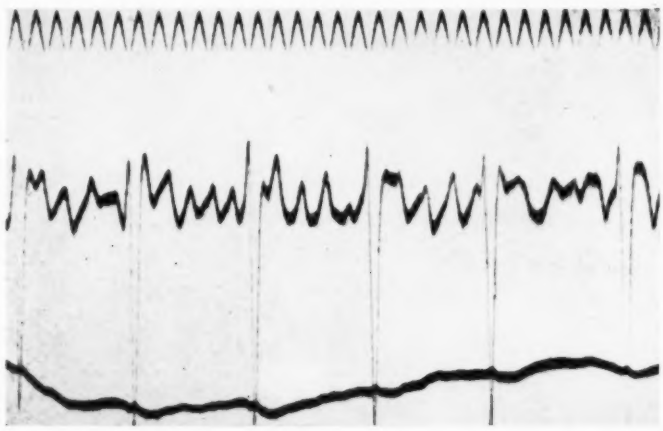
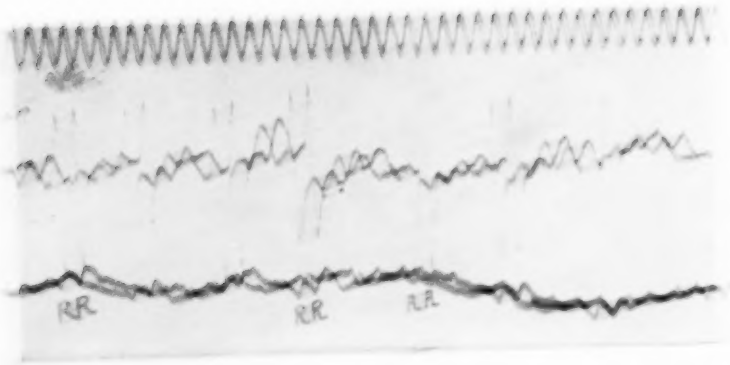


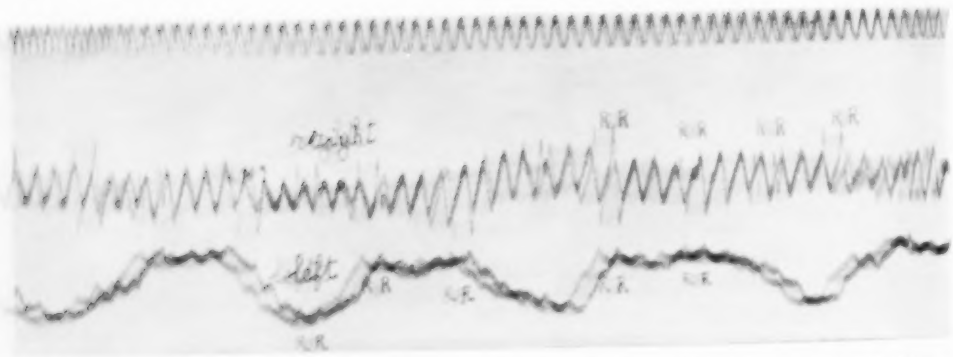
Fig. 6.—Flutter of the right auricle (upper curve) with complete absence of intrinsic electric waves in the left (lower curve). (?? standstill of the left auricle.) (From Luisada.[20])

We have already mentioned a possible purely technical explanation for this delay. The elongation of the muscle tracts in cases with great dilatation of the left auricle may also be a factor.

2. *Delay in contraction of the right auricle.* Rubino[11] and Luisada[19] have considered the possibility of this mechanism during ocular compression in a patient with permanent lengthening of the P-R interval. During the compression the "P *right*" wave was progressively delayed until it followed the "P *left*" wave (see Fig. 9).

The occurrence of auricular premature beats without change either of the ventricular rhythm or of the "P *left*"-R interval is another possible interpretation of these curves. Laufer and Rubino[9] observed also some cases in which the superimposition of the "P waves of the two auricles" suggested simultaneous contraction of the auricles. In such cases one may conjecture that the sino-auricular—right auricle conduction is so delayed that the excitation of the right auricle occurs only when the sinus impulse has already reached the left auricle.





CLINICAL INVESTIGATION OF AURICULAR DISORDERS

Scherf and Shookoff, Rothberger and Scherf, Condorelli, Scherf and Siedler, and others have studied intensively experimental disorders of the auricles; Groedel, Dressler, Mahaim, Condorelli, and many more have investigated clinical auricular disorders by means of the classical electrocardiographic leads. The new techniques already described have given a new stimulus for the solution of the problems of the following possible auricular disorders:

1. *Delay in contraction of the left auricle.* Condorelli,[1] Lauter and Rubinstein,[2] and Lasala[3] have repeatedly observed a delay of the left auricular contraction in hearts with enlargement of the left auricle in mitral disease, or with myocardial fibrosis due to coronary atherosclerosis or syphilitic stenosis of the coronary mouths. The delayed P wave arising from the left auricle may be either of normal height and form, or very small—as Rehm[4] and Sossai[5] have each described in a patient just after the change from auricular fibrillation to sinus rhythm. In cases with prolonged conduction from the sinauricular node to the left auricle, the classical leads show large, double, or diphasic P waves.

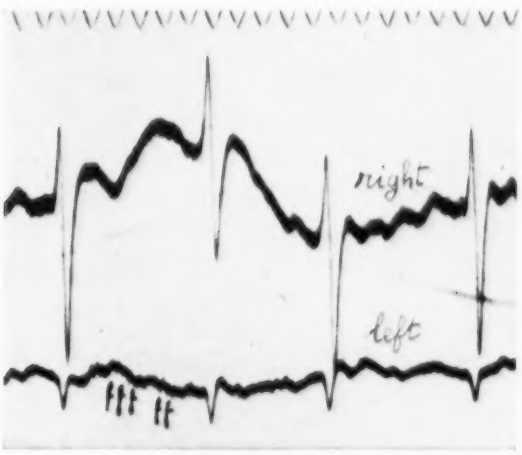


Fig. 5.—Fibrillation of both auricles. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. (From Luisada.[9])

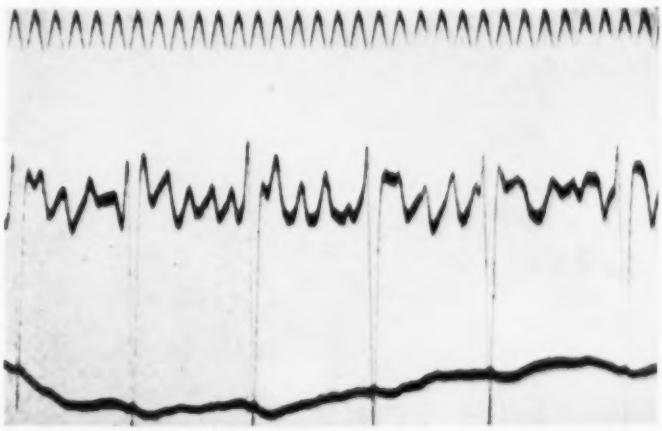


Fig. 6.—Flutter of the right auricle (upper curve) with complete absence of intrinsic electric waves in the left (lower curve). (77 standstill of the left auricle.) (From Luisada.[9])

We have already mentioned a possible purely technical explanation for this delay. The elongation of the muscle tracts in cases with great dilatation of the left auricle may also be a factor.

2. *Delay in contraction of the right auricle.* Rubino[11] and Luisada[19] have considered the possibility of this mechanism during ocular compression in a patient with permanent lengthening of the P-R interval. During the compression the "P *right*" wave was progressively delayed until it followed the "P *left*" wave (see Fig. 9).

The occurrence of auricular premature beats without change either of the ventricular rhythm or of the "P *left*"-R interval is another possible interpretation of these curves. Laufer and Rubino[9] observed also some cases in which the superimposition of the "P waves of the two auricles" suggested simultaneous contraction of the auricles. In such cases one may conjecture that the sino-auricular—right auricle conduction is so delayed that the excitation of the right auricle occurs only when the sinus impulse has already reached the left auricle.

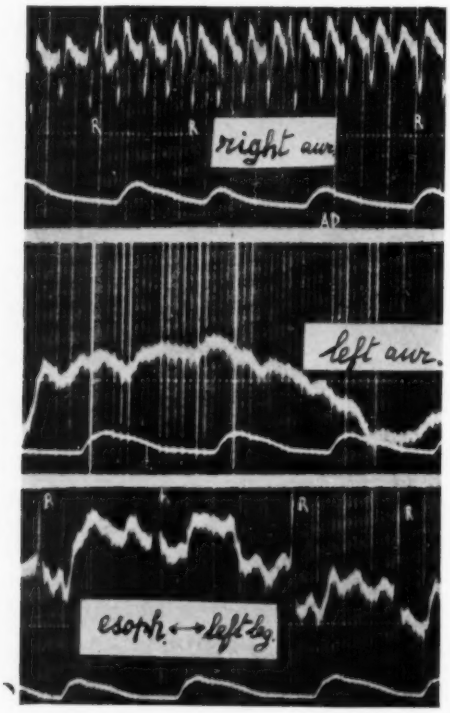


Fig. 7.—Flutter of the right auricle with fibrillation of the left. *A*, Lead for the right auricle (two-needle method) and brachial pulse (lower tracing). *B*, Esophageal lead for the left auricle and brachial pulse (lower tracing). *C*, Esophagus and left leg lead (it records the waves of both auricles). (From Sossai.[12])

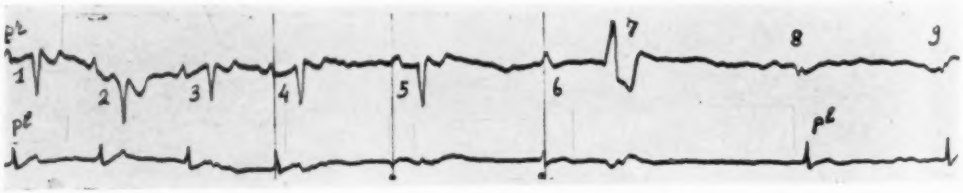


Fig. 8.—Effects of ocular compression in a vagotonic patient. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. The first four complexes are normal. *5* is an ectopic beat with inverted P left wave which precedes the P right wave. *6* is a sinus impulse reaching the left auricle and then the right (delayed conduction to the right auricle, block of impulse to the ventricles). *8* and *9* are two sinus discharges with normal P left waves, but with deformed P right waves and no ventricular complexes. (From Rubino.[11])

3. *Delay in contraction of both auricles with normal sinoauricular-atrioventricular node conduction and resulting decrease in P-R interval.* Condorelli[21] first established this conception experimentally by means of ligatures in the auricular muscle; later he, Laufer and Rubino,[9] and I[20] thought we found it clinically. Since, however, the electrocardiograph cannot register in man the moment of the sinus discharge, the clinical occurrence of this phenomenon cannot be proved. Two observations support the likelihood of this delayed conduction through the sinoauricular-auricular bundles when the sinoauricular-atrioventricular node conduction is normal or less delayed: first, the shortened P-R interval seen in patients with diffuse myocardial fibrosis with or without

bundle branch block; and second, the shortening of the P-R interval in a vagotonic subject during ocular compression.*

4. *Conduction block to ventricles and left auricle.* Rubino[11] referred to this possibility after recording esophageal records in a vagotonic patient during compression of the eyeballs. As indicated by the premature appearance of a slightly deformed auricular P wave, an ectopic beat arose in the right auricle, but no P wave of the left auricle and no ventricular complex followed. Artifacts can probably be excluded by the firm placing of the esophageal sound and by the reappearance of normal "P *left*" waves after several cardiac contractions.

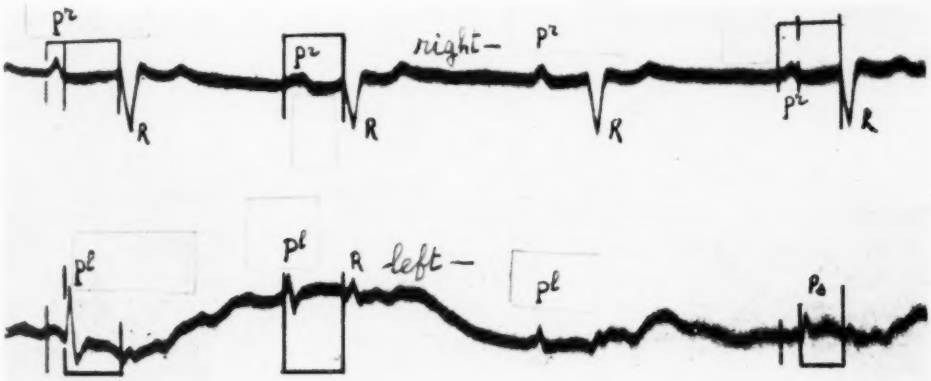


Fig. 9.—Effect of ocular compression in a vagotonic patient. Upper curve, lead for the right auricle (two-needle method). Lower curve, esophageal lead for the left auricle. The first complex shows a normal auricular and ventricular sequence (P right wave, P left wave, normal P-R interval). The second complex shows deformed synchronous P waves from both auricles and normal P left-R interval (slower conduction to the right auricle). The fourth complex shows deformed P waves with normal succession P right-P left, but shortened P-R interval (apparently slower conduction to both auricles with normal sinoauricular node-auriculoventricular conduction). (From Luisada.[20])

5. *Auricular premature beats without ventricular contraction.* Rubino[11] observed this in the same patient (in 4 above). Following total cardiac standstill induced by ocular compression, there occurred a series of "P *left*" waves, preceded, accompanied, or followed by "P *right*" waves, with no evidence of ventricular activity. When the "P *left*" waves fell earlier, or the complexes were deformed (or both occurred), it seems probable that the auricular complex was of ectopic origin. The oculocardiac reflex blocked the conduction of sinoauricular impulses to the ventricles.

6. *Flutter of the right auricle with fibrillation of the left.* Open chest experiments on dogs have demonstrated the possibility of dissociated rhythms in the two auricles. By ligating coronary vessels, Condorelli[21] has produced fibrillation of the left auricle while the right auricle beats normally.

Scherf and Siedeck,[27] by ligating muscle bundles, have produced many interesting disorders: (a) independent rhythms in the two auricles, either (1) a slow regular rhythm of the right auricle under the probable control of the

*Since vagal stimulation acts to delay conduction through the myocardium, it is not reasonable to assume that its physiologic action is reversed here. A preferable explanation is that the P-R interval is shortened because the sinoauricular-auricular conduction is delayed relatively more than the sinoauricular-atrioventricular node conduction and the conduction in the His bundle.

A-V node, with normal beating of the left auricle under the control of the sino-auricular node; or (2) the reverse of this, with sinus control of the right auricle and probable A-V nodal control of the slower left auricle; (b) fibrillation in the left auricle while the right auricle beats normally; (c) fibrillation in the right auricle while the left auricle beats forcibly but irregularly.

Many dissociated auricular rhythms have been described in man. So far, however, only flutter of the right auricle with either fibrillation or standstill of the left has been apparently indicated by the new techniques.

Laufer,[4] and later Laufer and Rubino,[9] Rubino,[10] Sossai,[12] and Luisada[20] repeatedly reported this interesting disorder.* In it, the "right auricular" curve shows irregular or regular high deflections, while a simultaneous lead from the left auricle shows frequent tiny deflections of the base line or total absence of intrinsic activity. Rubino,[10] and later Sossai,[12] each reported a remarkable case with this disorder, showing, on resumption of sinus rhythm, small, delayed "P *left*" waves.

So far, this disorder has been recorded nearly exclusively in patients with mitral stenosis in failure, being apparently favored by unequal distention of the two auricles. Sossai's case, for example, showed an aneurysm of the left auricle; when this decreased in volume following digitalization, the normal sinus rhythm resumed. In all these cases the peripheral leads have shown flutter-fibrillation (impure flutter). As with the cases of delay in contraction of the left auricle, these cases provide good opportunity for comparison of methods of auricular registration, for only the best methods can separate the two rhythms.

7. *Fibrillation or flutter of both auricles.* The occurrence of the same rhythm in both auricles is the usual observation; this may be fibrillation, flutter, or the relatively less common flutter-fibrillation (Rubino[10]). As indicated, impure flutter or tremulation, as recorded in classical leads, is caused by flutter in one auricle and fibrillation in the other, as well as by this flutter-fibrillation in both auricles. Fibrillation of both auricles occurs in patients either with mitral valve disease, or with myocardial fibrosis; at times, however, the greatly distended left auricle produces no deflections in the esophageal lead. A true paralysis of the left auricle could explain this, but mechanical methods are insufficiently delicate to prove it. Any auricular activity in the more normal right auricle may be poorly shown in the distended abnormal left auricle, even though the mechanism may be fundamentally the same in both.

DISCUSSION

Methods now available for registering auricular activity have added much to the interest of auricular disorders. Records taken with routine leads had suggested some of these, such as the delayed contraction of the left auricle. Other disorders, as dissociation between the auricles, with flutter in the right and fibrillation in the left, were wholly unsuspected, but now seem likely. Future use of the new methods may well reveal further unusual mechanisms. New theories advanced on the basis of open chest experiments may soon find

*A new case was described recently by Deglaude and Laubry.[15]

clinical confirmation. It is fundamentally important meanwhile to learn whether chest leads can ever show independent auricular action, that is, either right or left, which appears at present unlikely, and also to learn just how much of the total left and right auricular activities is shown by bipolar esophageal leads, still a moot point.

The classical theories dealing with the disturbed physiology causing auricular fibrillation and flutter will probably be revised in the future, in order to explain the dissociated auricular disorders. It is necessary, however, that more data be collected and statistical studies be made before accomplishing such a difficult task. Much remains for investigation; for example, the occurrence of varying rhythms in the two auricles. The cases with small, rapid, irregular deflections discussed by De Castro[22] and by Dagnini[23] as examples of "partial auricular fibrillation" (although obtained by an inadequate technique) may perhaps fall into this group and should be confirmed by the new methods.

The occurrence of *standstill of both auricles*, demonstrated by White[24] by both electrical and mechanical methods, and since confirmed by others (Levine[25]), has not yet been investigated by means of the new techniques. Neither has the occurrence of *alternate excitation of the right and the left auricle* (Lian and Golblin[26]; Scherf and Siedeck[27]), as suggested in the classical leads.

It must not be concluded from the foregoing observations that the two auricles are completely separate functional entities, although at times they may react independently in their properties of conductivity and contractility. True dissociation of the two auricles occurs only when the usual conduction pathways are interrupted by anatomic blocks, such as Condorelli, Scherf and Siedeck, and others produced in open chest experiments.

SUMMARY

1. Methods for the simultaneous and separate study of electrical activity in the auricles are reported.

2. The possibility of various bizarre disorders of auricular action in man, suggested by these methods, is discussed. Such possible disorders are: (a) delay in contraction of one auricle (right or left); (b) delay in contraction of both auricles with normal sinoauricular-auriculoventricular node conduction and resulting decrease in P-R interval; (c) conduction block to ventricles and left auricle; (d) auricular premature beats without ventricular contraction; (e) flutter of the right auricle with fibrillation of the left; (f) fibrillation, flutter, or flutter-fibrillation of both auricles.

3. Further possibilities of study of auricular disorders and the necessity of new interpretations are pointed out.

4. The experimental demonstration in animals of functional pathways through the auricular muscle needs further study in man in the clinic.

REFERENCES

1. Lewis, T.: Auricular Fibrillation and Its Relationship to Clinical Irregularity of the Heart, Heart **1**: 306, 1910.

2. Ackermann, R.: Thoracic Leads in Electrocardiography, Deutsches Arch. f. klin. Med. **144**: 61, 1924.

3. Condorelli, L.: Atti d. Soc. ital. per il progresso d. sc., Milano **12**: 18, 1931.
4. Laufer, S.: Zum Studium der Reizleitungsstörungen im Vorhof mittels direkter Ableitung des Vorhofelektrokardiogramms, Ztschr. f. klin. Med. **127**: 678, 1935.
5. Laufer, S.: Dérivations éléctives des oreillettes dans le diagnostic des troubles de la fonction auriculaire, Arch. d. mal. du coeur. **28**: 98, 1935.
6. Lian, C., Merklen, F. P., and Odinet, J.: Les dérivations précordiales en éléctrocardiographie, Arch. d. mal. du coeur. **27**: 189, 269, 1934.
7. Lieberson, A., and Lieberson, F.: Internal Electrocardiographic Lead, Proc. Soc. Exper. Biol. & Med. **31**: 441, 1934.
8. Luisada, A.: Derivazioni elettive per le correnti di origine atriale, Cuore e circolaz. **19**: 77, 1935.
9. Laufer, S., and Rubino, A.: Sull 'importanza delle derivazioni elettive nella diagnosi dei disturbi di conduzione del miocardio atriale, Clin. med. ital. **67**: 365, 1936.
10. Rubino, A.: Fluttuazioni e fibrillazioni parziali degli atri documentate col sistema delle derivazioni elettive, Cuore e circolaz. **20**: 57, 1936.
11. Rubino, A.: Blocco di emergenza, blocco interatriale e blocco atrio-ventricolare provocati a mezzo di stimolazione vagale in un caso con allungamento del tratto P-R, Clin. med. ital. **67**: 168, 1936.
12. Sossai, A.: Ricerche cliniche sulla specificità della derivazione esofagea per la registrazione dell'elettrogramma dell'atrio sinistro, Cuore e circolaz. **22**: 441, 1938.
13. Brown, W. H.: Study of the Esophageal Lead in Clinical Electrocardiography, Am. Heart J. **12**: 307, 1936.
14. v. Zàrday, I.: Klinische Erfahrungen mit elektrokardiographischen Brustwandableitungen, Arch. f. Kreislaufforsch. **2**: 157, 1938.
15. Deglaude, L., and Laubry, P.: L'électrocardiogramme oesophagien, Arch. d. mal. du coeur. **32**: 121, 1939.
16. Spühler, O.: Das Oesophagus-Elektrokardiogramm, Ztschr. f. klin. Med. **134**: 671, 1938.
17. de Chatel, A., and Hussey, R.: Experimentelle Untersuchungen über das Elektrokardiogramm bei Ableitung vom Oesophagus, Ztschr. f. klin. Med. **131**: 450, 1937.
18. Luisada, A., Rubino, A., and Canella, C.: L'elettrogramma dell'atrio destro e del seno cardiaco, Cardiol. **1**: 280, 1937.
19. Kossmann, C. E., and Rader, B.: The Effect of Potential Variations of the Distant Electrode on the Precordial Electrocardiogram, Am. Heart J. **12**: 698, 1936.
20. Luisada, A.: Cardiologia—patologia e clinica apparato circolatorio, Bologna, 1938, Cappelli.
21. Condorelli, L.: Ueber die Bahnen der Reizleitung vom Keith-Flackschen Knoten zu den Vorhöfen, Ztschr. exper. Med. **68**: 493, 1929; Ricerche sperimentali sulla conduzione sino-nodale, Cuore e circolaz. **14**: 221, 1930; Experimentelle Untersuchungen über die interaurikulare Reizleitung, Ztschr. exper. Med. **68**: 516, 1929.
22. De Castro: Flutter et fibrillations partielles, Arch. d. mal. du coeur. **27**: 282, 1934; Nouveaux aspects de la pathologie auriculaire, Arch. d. mal. du coeur. **30**: 207, 1937.
23. Dagnini, G.: L'attività auricolare prefibrillatoria, Cuore e circolaz. **21**: 571, 1937.
24. White, P. D.: Auricular Standstill: An Unusual Effect of Digitalis on the Heart, With Especial Reference to the Electrocardiogram, Boston M. & S. J. **177**: 233, 1916.
25. Levine, S.: N. E. Heart Ass. Meeting, March, 1939.
26. Lian, C., and Golblin, V.: Un cas de double rythme auriculaire par dissociation interauriculaire, Arch. d. mal. du coeur. **31**: 52, 1938.
27. Scherf, D., and Siedeck, H.: Ueber Block zwischen beiden Vorhöfen, Ztschr. f. klin. Med. **127**: 77, 1934.

A MICROMETHOD FOR DETERMINATION OF TISSUE LIPIDS*

E. L. Outhouse, M.A., and J. C. Forbes, M.A., Ph.D., Richmond, Va.

THE need for a convenient and accurate method for the determination of the lipid content of tissue has long been recognized. Several methods of lipid analysis have been developed. These methods give satisfactory results, but do not quite fill the requirements for routine analysis of a large number of samples, as they are time-consuming and laborious.

During the course of the past two years we have developed and used a method of lipid analysis which we find to be a marked improvement over the procedures in general use. This method saves time, requires only simple apparatus, and gives results that agree with those obtained by accepted procedures. Essentially it is the extraction method of Forbes and Irving[1] for the estimation of cholesterol in serum, applied to the extractions of the neutral fat and cholesterol of tissue, with the technique of Bloor and Snider[2] for the preparation of the extract for estimation of phospholipids. The estimation of neutral fat and cholesterol is made by the application of Bloor's[3] oxidative method to the chloroform-doucil extract. Total cholesterol is determined by the Leibermann-Burchard reaction, and the free cholesterol by the Sturges and Knudson[4] modification of the Schoenheimer-Sperry method.[5]

Phospholipids are calculated from the determination of the phosphorus in the alcohol-ether extract, or the phosphorus of the moist ether fraction, depending upon whether it is desirable to determine the total phospholipid or the lecithin-cephalin phospholipid. The sphingomyelin content of liver may be obtained from the difference between these two values (Kirk[6]).

PREPARATION OF THE TISSUE EXTRACTS

Immediately after decapitating and draining the blood from the animal, the liver is removed and ground in a mortar. Thorough grinding is necessary, particularly when the livers are fatty, because the fat is frequently unevenly distributed.

PREPARATION OF ALCOHOL-ETHER EXTRACT

One to 1.5 Gm. of finely ground liver is weighed on an analytical balance and transferred to a mortar containing 5 Gm. of fine sand. It is ground thoroughly and transferred with a spatula to a 125 c.c. Erlenmeyer flask. The mortar and pestle are washed twice with 20 c.c. of alcohol-ether (3:1) each time, the washing being added to the flask. The liver and sand mixture is thoroughly ground by stirring with a flat-tipped stirring rod. After an hour the flask is placed in a 70° C. water bath and allowed to boil for about two minutes. The alcohol-

*From the Department of Biochemistry, Medical College of Virginia, Richmond.
Received for publication, October 2, 1939.

ether extract is filtered through a rapid filter paper into a 100 c.c. volumetric flask, and the residue is re-extracted twice with 25 c.c. of alcohol-ether as before. A final extraction is made with 20 c.c. of alcohol-ether at room temperature. When filtering is complete, the alcohol-ether is added to the 100 c.c. mark. The extract is shaken thoroughly, and an aliquot is taken for phospholipid determination.

PREPARATION OF THE DOUCIL-CHLOROFORM EXTRACT

One to 1.5 Gm. of the finely ground liver is placed in a mortar containing 5 Gm. of doucil. The tissue and doucil are ground to a very fine powder and transferred to a 125 c.c. glass-stoppered pyrex bottle, and 100 c.c. of chloroform are added. The bottle is stoppered and shaken thoroughly. The shaking is repeated a number of times during the next few hours, and the extract is allowed to stand overnight. After thorough shaking the extract is filtered through a fat-free filter paper into another glass-stoppered bottle. The extract is now ready for use.

The advantage of this extract is that it contains only the neutral fat, cholesterol, and cholesterol esters of the tissue. A series of determinations has demonstrated that the phospholipids are completely removed by the doucil. This is of particular advantage in the colorimetric estimation of cholesterol by the Leibermann-Burchard reaction, as interference by phospholipids has been a criticism of this method of cholesterol estimation.

DETERMINATION OF LIPIDS

Neutral Fat Plus Cholesterol.—An aliquot of the chloroform extract, 1 c.c. to 5 c.c., depending upon the fat content of the liver, is measured into a 125 c.c. glass-stoppered pyrex flask. The flask is then placed in a water bath at 70° C., and all traces of solvent removed by a current of air. The neutral fat plus cholesterol residue is then determined by Bloor's[3] oxidative technique. Since the oxidation value per milligram of triglyceride in liver so closely approximates that of the fatty acids themselves, we have used Bloor's empirical relationship of 3.70 c.c. of 0.1 N sodium thiosulfate as equivalent to 1.0 mg. of lipid.

Total Phospholipid.—A 5 c.c. aliquot of the alcohol-ether extract is placed in a 30 c.c. pyrex micro-Kjeldahl flask. The flask is then suspended in a 70° C. water bath, and the solvent is removed by a current of air. Care must be taken that all traces of solvent are removed, as the presence of the solvent gives difficulty in the digestion for estimation of phosphorus. The phosphorus is determined by a slight modification of King's[7] method for total phosphorus.

After removal of the last traces of solvent from the sample, 0.1 c.c. of nitric acid is added, followed by 1.2 c.c. of 60 per cent perchloric acid. The flask is placed on a digestion heater, preferably an electric one, and digested until the digestion mixture is clear. The heat should be so regulated that the digestion takes approximately twenty minutes. If the fat content of the liver is high, the digestion should be continued for thirty minutes.

After digestion the flasks are permitted to cool, and the contents are transferred to tubes graduated at 15 c.c. The contents of the digestion flask are

diluted to about 5 c.c. and decanted into the graduated tube. The flask is washed three times with about 2 c.c. of water, bringing the total volume in the tube to about 11 c.c. A standard tube containing 0.1 mg. of phosphorus is made up by taking 10 c.c. of the phosphorus standard and adding 1 c.c. of 60 per cent perchloric acid. If the phosphorus is low, it is advisable to make up another standard containing 0.05 mg. of phosphorus. To the standard and unknown 1 c.c. of 5 per cent ammonium molybdate is added, followed by 1 c.c. of 0.1 per cent aminonaphtholsulfonic acid solution. Water is then added to the 15 c.c. mark, and the solution is mixed thoroughly by inverting the tube several times. After standing for five minutes, the unknown is compared with the standard in a colorimeter. The total phospholipid is calculated by multiplying the phosphorus value by 25.

Lecithin-Cephalin Phospholipid.—Twenty cubic centimeters of the alcohol-ether extract are placed in a 50 c.c. Erlenmeyer flask and evaporated to dryness on a 70° C. water bath with a current of air. After removing from the water bath, 0.3 c.c. of water is added, followed by a few drops of moist ether. The flask is thoroughly shaken and the ether is evaporated off. Ten cubic centimeters of moist ether are added. The flask is shaken and placed in a water bath until the ether boils and the volume is reduced to 5 c.c. The moist ether solution is decanted into a 25 c.c. glass-stoppered graduated cylinder, care being taken that none of the aqueous layer is transferred. The extraction is repeated twice, thus making a total volume in the cylinder of between 15 and 20 c.c. Moist ether is added to the 20 c.c. mark. The flask is stoppered, shaken thoroughly, and allowed to stand overnight. A slight cloudy precipitate settles out. Without disturbing the precipitate, a 5 c.c. aliquot is removed, and the phosphorus content is determined as described for total phospholipid.

The sphingomyelin content of the tissue can be determined by the difference between the total and the lecithin-cephalin phospholipid.

Total Cholesterol.—To 5 c.c. of the chloroform extract in a 10 ml. glass-stoppered cylinder are added 2 c.c. of a 20:1 mixture of acetic anhydride with concentrated sulfuric acid. The sulfuric acid should be added to the acetic anhydride, shaken thoroughly, and cooled to 25° C. immediately before use. It has been found that much better results are given by this method than by the usual procedure of adding 0.1 c.c. of sulfuric acid to the chloroform acetic anhydride mixture. After adding the acetic anhydride-sulfuric acid mixture, the cylinders are stoppered, shaken thoroughly, placed in a beaker of water at 25° C., and put in the dark for ten to fifteen minutes for color development. They are then compared in a Duboscq colorimeter with a standard containing 0.2 mg. of cholesterol prepared at the same time and under the same conditions. A Wratten filter No. 71A and a permanent standard, as recommended by Shapiro, Lerner, and Posen,[8] are used for the comparison of color.

Free Cholesterol.—A 5 c.c. aliquot of the chloroform extract is placed in a 15 c.c. centrifuge tube and the chloroform removed by placing it in a 70° C. water bath and directing a current of air on the surface of the liquid. Bumping may be prevented by placing a small stirring rod in the tube. When the chloroform has been completely removed, 1 c.c. of alcohol-acetone (1:1) solution

is added. The lipid is dissolved by stirring. Three drops of 5 per cent hydrochloric acid and 1 c.c. of 0.3 per cent digitonin in 90 per cent alcohol are added. The stirring rod is removed and placed so that it can be returned to its tube; the tube is centrifuged fifteen minutes at 2,000 r.p.m. The supernatant liquid is decanted and the precipitate and stirring rod are washed with acetone-ether (1:2), the centrifuging is repeated, and the supernatant liquid is removed by suction. The washing is repeated twice with anhydrous ether.

The last trace of ether is removed with a current of dry air on a water bath, and the cholesterol digitonide is dissolved in 1 c.c. of acetic acid by placing the tube in a 60° C. water bath. After removing from the bath and cooling, 2 c.c. of acetic anhydride-sulfuric acid (20:1) mixture, as described for total cholesterol, is added, and the tube is placed in a 25° C. bath and kept in the dark for twenty-five minutes. It is compared with a standard containing 0.2 mg. of cholesterol, prepared by treating a standard cholesterol solution in chloroform exactly as above. The standard cholesterol and the unknown are usually set at 10 mm., and the readings are taken of the permanent standard.

Typical results obtained by the methods here described are compared with those obtained with well-established methods in Tables I, II, and III.

DISCUSSION

That Bloor's oxidative method of determining the total fatty acids and cholesterol of tissue can be applied to the chloroform extract is indicated by the close agreement shown in Table I between the values obtained by Bloor's method on the alcohol-ether extract and those obtained by calculating these constituents from the values obtained from the fatty acids of the total phospholipid, and the neutral fat plus cholesteroi of the chloroform extract.

TABLE I

COMPARATIVE DETERMINATIONS OF TOTAL FATTY ACIDS PLUS CHOLESTEROL IN NORMAL AND FATTY LIVERS OF RATS

The values are expressed as per cent of fresh tissue

TOTAL PHOSPHOLIPIDS	NEUTRAL FAT PLUS CHOLESTEROL	TOTAL FATTY ACIDS PLUS CHOLESTEROL	
		AUTHORS' METHOD	BLOOR'S METHOD
3.30	23.8	25.1	25.1
3.04	28.0	28.8	29.2
2.79	21.8	22.7	23.4
2.65	32.2	32.6	33.2
3.92	4.5	7.0	6.4
4.10	5.8	8.4	8.4
3.02	14.5	16.0	16.6
3.66	7.6	9.8	9.7
2.42	29.0	29.4	31.2
2.20	35.0	35.0	35.4
3.60	8.3	10.4	9.9
3.12	13.6	15.2	15.2
2.70	29.3	29.9	28.6
3.58	0.89	3.3	3.3
3.41	0.92	3.2	3.1
3.54	0.90	3.3	3.5
3.22	0.74	2.9	3.0
3.56	0.89	3.4	3.4
2.79	1.10	3.0	3.0
3.54	0.72	3.1	3.0

TABLE II

COMPARATIVE DETERMINATIONS OF FREE AND TOTAL CHOLESTEROL IN NORMAL AND FATTY LIVERS OF RATS

The values are expressed as per cent of fresh tissue

TOTAL CHOLESTEROL			FREE CHOLESTEROL	
DOUCIL-$CHCl_3$ EXTRACT		ALCOHOL-ETHER EXTRACT	DOUCIL-$CHCl_3$ EXTRACT	ALCOHOL-ETHER EXTRACT
AUTHORS' METHOD	DIGITONIDE METHOD	DIGITONIDE METHOD	DIGITONIDE PRECIPITATION	DIGITONIDE PRECIPITATION
0.19	0.19	0.19	0.17	0.17
0.20	0.21	0.23	0.19	0.19
0.24	0.23	0.24	0.17	0.18
0.19	0.19	0.20	0.16	0.15
0.22	0.22	0.22	0.20	0.20
0.25	0.27	0.22	0.21	0.20
0.21	0.21	0.20	0.16	0.16
1.15	1.15	1.14	0.22	0.22
0.41	0.44	0.46	0.22	0.24
0.90	0.90	0.94	0.24	0.25
0.89	0.87	0.88	0.17	0.19
0.67	0.65	0.69	0.17	0.19
0.36	0.34	0.35	0.18	0.19
0.77	0.76	0.80	0.16	0.18
0.87	0.86	0.90	0.21	0.23
0.38	0.38	0.40	0.22	0.23
0.87	0.87	0.81	0.21	0.20

Note.—The digitonide method used is a slight modification of Schoenheimer and Sperry's method.

TABLE III

TYPICAL RESULTS OF COMPLETE LIPID ANALYSIS OF RAT LIVERS

The values are expressed as per cent of fresh tissue

TOTAL PHOSPHO-LIPID	LECITHIN-CEPHALIN	SPHINGO-MYELIN	NEUTRAL FAT AND CHOLES-TEROL	TOTAL CHOLES-TEROL	FREE CHO-LESTEROL	ESTER CHOLES-TEROL	IODINE NUM-BER OF NEU-TRAL FAT AND CHO-LESTEROL
2.31	1.92	0.39	33.3	0.54	0.15	0.39	88
3.34	2.67	0.67	12.1	0.51	0.18	0.33	94
3.35	2.69	0.66	0.9	0.20	0.18	0.02	117

Repeated extractions with moist ether of the residues from evaporation of the chloroform extracts of a large number of livers has demonstrated that the chloroform extract contains no trace of phospholipids. Only neutral fat, cholesterol, and cholesterol esters are present in this extract.

Completeness of extraction is demonstrated by the agreement between the total fatty acid values on the two extracts, as given in Table I, and also by the agreement of the cholesterol values in Table II, as determined by the digitonide method on the chloroform extract and the alcohol-ether extract.

The determination of phospholipid by calculation from the phospholipid phosphorus is in general use, and according to Kirk[6] can be used for determining total phospholipid (alcohol-ether soluble phospholipid), lecithin and cephalin (moist ether-soluble phospholipids), and sphingomyelin (phospholipid soluble in alcohol-ether and insoluble in moist ether).

There are a number of methods in use for the determination of cholesterol. Criticisms of the methods of cholesterol determination are so common that there

seems to be no necessity of detailed discussion of the merits and demerits of the various procedures. The Schoenheimer-Sperry method, which appears to be the method in common use at present, was chosen as a standard for evaluating our procedure for determination of free and total cholesterol. As shown in Table II, the method described for estimation of free and total cholesterol gave values that agree with those obtained by the Schoenheimer-Sperry method.

Some difficulty was experienced in the precipitation of the cholesterol digitonide, in the determination of total cholesterol, when applying the Schoenheimer-Sperry method to fatty livers. Frequently the total cholesterol of fatty livers, produced either by carbon tetrachloride poisoning or by a high fat diet, gave values lower than those obtained for free cholesterol. Results could not be duplicated, and commonly ranged from 10 per cent to 60 per cent of the value obtained for total cholesterol by the Leibermann-Burchard reaction applied to the chloroform extract. Sturges and Knudson[4] reported similar experiences and suggested that this could be overcome by adding 3 drops of 5 per cent hydrochloric acid in excess after neutralizing the solution following saponification. They used only normal tissues in their experiments. We found that this procedure would give good results on normal livers, but made no improvement when the method was applied to fatty livers. The substitution of 1 drop of 50 per cent sodium hydroxide for the potassium hydroxide in the saponification solved this difficulty, and gave satisfactory duplicate results at all times. Furthermore, these results have always agreed with those obtained using the method described in this paper.

The chloroform extract has been found to be suitable for determination of iodine numbers by the method of Yasuda.[9]

SUMMARY

1. The preparation of a chloroform extract of liver suitable for the determination of neutral fat, total cholesterol, free cholesterol, ester cholesterol, and iodine number of the neutral fat plus cholesterol, is described.

2. A convenient method for the determination of total phospholipids, lecithin-cephalin phospholipids, and sphingomyelin content of liver is presented.

We gratefully acknowledge their great indebtedness to the John and Mary R. Markle Foundation for a grant in aid of the research.

We are also indebted to Miss Lui Phipps for her assistance in checking many of the cholesterol determinations.

The doucil, a synthetic zeolite, was kindly supplied by the W. A. Taylor Company, Baltimore, Md.

REFERENCES

1. Forbes, J. C., and Irving, H.: Determination of Cholesterol in Blood Plasma and Serum. J. Lab. & Clin. Med. **16**: 520, 1931.
2. Bloor, W. R., and Snider, R. H.: Phospholipid Content and Activity in Muscle, J. Biol. Chem. **107**: 459, 1934.
3. Bloor, W. R.: The Determination of Small Amounts of Lipid in Blood Plasma, J. Biol. Chem. **77**: 53, 1928.
4. Sturges, S., and Knudson, A.: Application of the Schoenheimer-Sperry Method to the Determination of Cholesterol and Cholesterol Esters in Tissues, J. Biol. Chem. 126: 543, 1938.
5. Schoenheimer, R., and Sperry, W. M.: A Micromethod for the Determination of Free and Combined Cholesterol, J. Biol. Chem. **106**: 745, 1934.

6. Kirk, E.: A Micromethod for Approximate Estimation of Lecithin, Cephalin, Ether-Insoluble Phosphatide, and Cerebrosides in Plasma Red Blood Cells and Tissues, J. Biol. Chem. **123**: 623, 1938.
7. King, E. J.: The Colorimetric Determination of Phosphorus, Biochem. J. **26**: 292, 1932.
8. Shapiro, A., Lerner, H., and Posen, E.: A Fixed Color Standard for Cholesterol Determinations, Proc. Soc. Exper. Biol. & Med. **32**: 1300, 1935.
9. Yasuda, M.: The Determination of the Iodine Number of Lipids, J. Biol. Chem. **94**: 401, 1931.

FATAL REACTIONS TO ADMINISTRATION OF SULFONAMIDE DRUGS*

Report of Five Cases

L. J. Tragerman, M.D., and J. M. Goto, M.D., Los Angeles, Calif.

IN THE wake of numerous successful and life-saving results from the use of the sulfonamide drugs, there continue to appear reports and mention of occasional fatal reactions. So far these reports do not seriously challenge the use of these compounds, but serve to warn against the use of such potent agents without adequate indication or supervision. The reported fatal complications have been granulocytopenia, hemolytic anemia, and liver damage. Since the introduction of these drugs, we have encountered examples of each of these complications in our autopsy service at the Los Angeles County Hospital. To increase slightly the fund of available information concerning fatal reactions, five deaths with autopsy are reported. Three of the patients died from granulocytopenia, one from apparent liver and kidney damage, and one from hemolytic anemia. In four patients sulfanilamide was the drug used. In the fifth patient, who died of granulocytopenia, sulfapyridine was used.

FATAL GRANULOCYTOPENIA INDUCED BY SULFANILAMIDE

The most recent review of fatal granulocytopenia associated with sulfanilamide therapy[1] brought the total number of reported cases to ten. At about the same time, two additional cases were recorded by Ottenberg.[2] Other cases have been reported by Corr and Root,[3] Sailer,[4] and Garvin.[5] The following are summaries of the clinical records and necropsy findings in two additional cases.

Case 1.—(Los Angeles County Hospital, No. 635-179.) A 15-year-old school boy entered the hospital on November 2, 1938, with a scarlatinal rash and fever of one day's duration. Temperature was 100° F. and enanthem was noted in the pharynx. Urinalysis was negative and blood count revealed hemoglobin of 85 per cent (Sahli), erythrocytes 4.1 million, and leucocytes 12,500 per c. mm., with 85 per cent polymorphonuclear cells and 15 per cent lymphocytes. Sulfanilamide, 6.4 Gm. daily, was given for seven days until the temperature returned to normal. After two days without treatment a scarlatinal eruption recurred and sulfanilamide was again started and administered for the rest of the patient's stay in the hospital. The total amount given was 100.6 Gm. over an eighteen-day period. The day before dis-

*From the Department of Pathology, University of Southern California School of Medicine, and the Laboratories of the Los Angeles County Hospital.
Received for publication, October 7, 1939.

charge the patient's blood count showed 2.6 million erythrocytes and 5,030 leucocytes per c. mm., with 74 per cent polymorphonuclear cells, 24 per cent lymphocytes, and 2 per cent monocytes. The blood sulfanilamide level was 5.5 mg. per 100 c.c. on this day.

After discharge the patient felt well and took no other drugs, but within three days he was again acutely ill, with sore throat and temperature of 104° F. He re-entered the hospital on November 24, 1938. Mild hyperemia of the pharynx and reddened left eardrum were the only physical findings noted. His blood count, however, now showed hemoglobin of 60 per cent (Sahli), erythrocytes 4.0 million per c. mm., and leucocytes only 675 per c. mm., with no polymorphonuclears or monocytes. Blood culture, taken on entry, yielded no growth. Blood transfusion, leucocytic extract, and yellow bone marrow were administered with no improvement. The following day his hemoglobin was 58 per cent (Sahli), erythrocytes were 3.6 million per c. mm., and leucocytes 550 per c. mm. He died with no further change in his condition. The clinical diagnosis was granulocytopenia, probably induced by sulfanilamide.

At necropsy there was only slight gross evidence of pharyngitis. The paranasal sinuses, aural cavities, meninges, and brain were normal. A few small areas of partial consolidation were present in each lung. The liver weighed 2,050 Gm. and its cut surface had obscured markings and a peculiar salmon pink color frequently seen in the livers of patients who had received sulfanilamide shortly before death. Chemical analysis of a portion of the liver revealed 2.5 mg. per cent of free sulfanilamide (Table I). The spleen weighed 250 Gm. and its cut surface was soft, blue black, with obscured Malpighian markings. The bone marrow of the sternum, vertebral bodies, and the upper end of the femur was hyperemic.

Microscopic examination of the consolidated areas of the lungs showed hyperemia, intra-alveolar edema, occasional mononuclear cells, but no polymorphonuclear leucocytes. The liver showed moderate parenchymatous degeneration and slight fatty change. The glomeruli of the kidneys appeared unchanged. Many of the convoluted tubules showed severe cloudy swelling with some karyolysis. The splenic pulp was markedly hyperemic with striking diminution of granular leucocytes. Increased iron-containing pigment was demonstrated by the Prussian blue stain. Sections of the vertebral marrow and smears of the sternal marrow showed reduction in the granular series with maturation arrest at the myelocyte level. Erythroblasts were abundant and numerous plasma cells were also noted.

CASE 2.—(Los Angeles County Hospital, No. 398-057.) A Caucasian housewife, aged 35 years, after several recurrent attacks of sore throat early in September, 1937, was seen by a private physician, who made a diagnosis of "streptococcal sore throat" and started treatment with sulfanilamide. The patient failed to return to her physician for observation, but continued to take sulfanilamide to a total of 64 Gm. over a period of three weeks. She then entered the hospital because of failure to improve. She was acutely ill, with a temperature of 104.3° F., pulse 140, and respirations 24 per minute. The pharynx was obscured by a gray membrane from which no bacteria were obtained on smear. Laboratory findings included normal urine and blood urea nitrogen of 12 mg. per 100 c.c. The blood count revealed hemoglobin 7.5 Gm., erythrocytes 2.4 million, platelets 266,000, leucocytes 550 per c. mm., with 97 per cent lymphocytes, 3 per cent monocytes, and no polymorphonuclear neutrophiles. Treatment consisted of blood transfusions, pentonucleotide, and liver extract. The patient's temperature remained high until death on the third day after entry to the hospital.

At autopsy moderate jaundice and cervical lymphadenopathy were externally evident. The pharynx and tonsils were partially necrotic and covered with a yellow-gray membrane. In both lungs there were considerable hyperemia and edema. The liver weighed 2,200 Gm. Surfaces made by sectioning were softer than normal and had obscured markings. The spleen weighed 300 Gm. The pulp was red and softened, obscuring the normal markings. The kidneys together weighed 300 Gm. and showed cloudy swelling. The bone marrow of the sternum and bodies of the lumbar vertebrae and femur appeared grossly normal. Histologically, the necrotizing pharyngitis was characterized by an absence of polymorphonuclear leucocytes. In the liver there was parenchymatous degeneration associated with necrosis of scattered cells in the centers of some lobules. The spleen showed an increase of mononuclear elements. The bone marrow, unfortunately, was not examined microscopically.

COMMENT

It appears likely that the fatal granulocytopenia in these two patients is attributable to sulfanilamide. They resemble previously reported cases. The onset of granulocytopenia was noted about three weeks after treatment was instituted. In the second patient the dosage used was average. In the first patient the daily dosage (6.4 Gm.) was higher than that generally recommended by Long.[6] The total dosage used, 101 Gm., was twice the average in the reported cases of granulocytopenia, but it apparently was excreted adequately, since the blood level near the conclusion of treatment was only 5.5 mg. per cent. The continuance of sulfanilamide for a week or more after the subsidence of the acute symptoms, as was done in this patient, is considered good practice in the avoidance of complications following scarlet fever. In this connection, it is of interest to note that the leucocyte count of the blood at the cessation of treatment was only slightly below normal. It would appear, as with amidopyrine, that fatal granulocytopenia may evolve after discontinuing the use of the offending drug. With the second patient it is, of course, possible that agranulocytic angina was already present when sulfanilamide was started, since no early blood counts were available. The patient did not return to her physician, but the total dosage taken before entry to the hospital was not excessive. Another point, emphasized by others as a poor prognostic sign, is the absence of monocytosis during the period of granulocytopenia. This was also noted in both cases reported here. The iron pigmentation of the spleen has been noted both in reported cases and in experimental observations.[7] The bone marrow picture is similar to that already described in sulfanilamide granulocytopenia.[1]

FATAL GRANULOCYTOPENIA INDUCED BY SULFAPYRIDINE

The introduction of the new compound, sulfapyridine, was accompanied by the warning that toxic effects similar to those produced by sulfanilamide might be anticipated,[8] and case reports of granulocytopenia began to appear soon thereafter.[9-11] In at least four instances the granulocytopenia was fatal. Rosenthal and Vogel's[12] patients were children who received 95 and 35 Gm. of the drug for *Staphylococcus aureus* sepsis and lobar pneumonia, respectively, and developed granulocytopenia in about two weeks. Autopsy findings are not recorded. Dolgopol and Hobart[13] have reported a similar case in which fatal granulocytopenia complicated the treatment of type VII pneumococcic pneumonia with 49 Gm. of sulfapyridine. At autopsy severe depression of bone marrow was found. Johnston's[9] patient had been treated for acute *Streptococcus viridans* bacterial endocarditis complicating puerperal sepsis, with 54 Gm. of sulfapyridine over an eleven-day period. Neutropenia soon appeared and at necropsy, several days later, the endocarditis was still evident. The following is a report of a somewhat similar case.

CASE 3.—(Los Angeles County Hospital, No. 648-416.) C. B., a white male of 53, entered the hospital on February 16, 1939, with fever, weakness, and fleeting joint pains of five weeks' duration. Following a peritonsillar abscess one year previously, he had progressive weakness, cough, and weight loss. There was no past history of rheumatic fever. Physical findings included low-grade fever, regular pulse, rough systolic apical murmur, and palpably enlarged spleen. Laboratory examination revealed normal urine, blood count of 4.3

million erythrocytes per c. mm., 75 per cent hemoglobin (Sahli), and 7,500 leucocytes with 95 per cent polymorphonuclear cells. Several blood cultures were positive for *Streptococcus viridans*. The clinical diagnosis was subacute *Streptococcus viridans* endocarditis.

The patient was given 88.5 Gm. of sulfapyridine between February 22 and March 15, 1939. A temporary remission of fever was noted during the first few days of treatment. On March 2 the blood sulfapyridine was 8.2 mg. per 100 c.c., and the blood culture was positive for *Streptococcus viridans*. On this day blood count revealed hemoglobin of 60 per cent (Sahli), leucocytes 11,250 per c. mm., with 80 per cent polymorphonuclear cells and 30 per cent lymphocytes. No further blood count was done until March 15, when the patient complained of sore throat. Blood count now showed a hemoglobin of 50 per cent (Sahli), erythrocytes 2.7 million per c. mm., and leucocytes 1,100 per c. mm., with 78 per cent lymphocytes and 22 per cent polymorphonuclears. Sulfapyridine was immediately discontinued and blood transfusion was given. The anemia and leucopenia persisted, the last leucocyte count being 1,450 per c. mm. on the day of death (March 21, 1939).

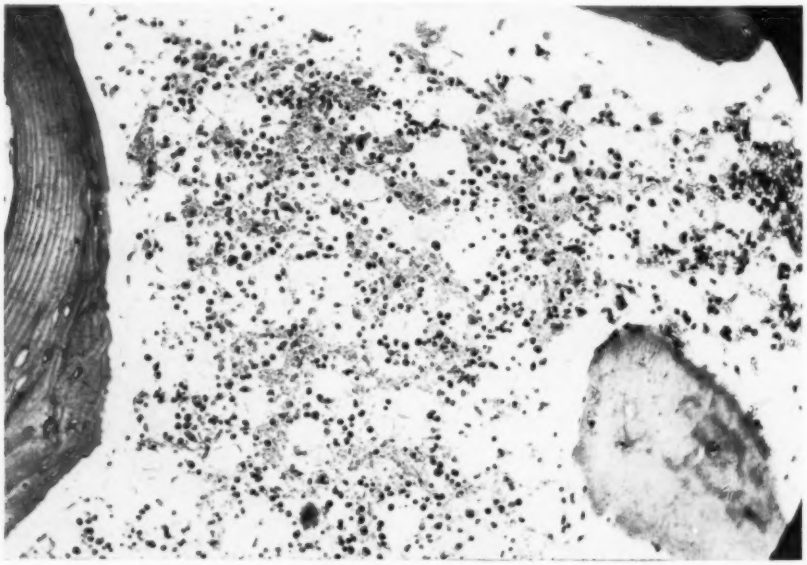


Fig. 1.—Vertebral bone marrow. Giemsa stain (×235). Fibrinoid necrosis and reduction of myeloid series is shown.

At necropsy numerous petechiae and moderate jaundice were noted in the skin and mucous membranes. No areas of softening were noted in the brain. The heart weighed 330 Gm. All chambers were somewhat dilated, the valve measurements were within normal limits, but the mitral leaflets were slightly thickened and shortened, and the tips of the papillary muscles were fibrosed. In addition, there were several small soft light brown vegetations on the anterior leaflet of the mitral valve. These were situated near the attachment of several of the chordae tendineae onto which they extended. None was over several millimeters in diameter. The liver appeared grossly normal. The spleen weighed 370 Gm. and showed, in addition to softened pulp, a recent infarct 2 cm. in diameter near one surface. The kidneys together weighed 370 Gm. and showed a few small infarcts. Apart from moderate hyperemia of the lungs and some small mucosal ulcerations in the esophagus, the other organs showed no striking alteration. The bone marrow of the sternum, vertebral bodies, and long bones was grossly normal.

Histologic examination showed several changes of interest. Section made through the anterior mitral leaflet in the region of the small gross vegetation showed moderate thickening of the valvular endocardium, with scattered fibrinous deposits on the surface. In some of these areas bacterial masses were seen. In the subendocardial tissue there were occasional

collections of mononuclear cells about small thickened blood vessels. In the section taken through the area of gross infarction in the spleen there were numerous clusters of bacteria, generally in blood vessels within the infarcted area. No wall of leucocytes occurred at the margin of the infarct, and polymorphonuclears were generally scarce throughout the uninvolved portions. Large quantities of iron pigment were also demonstrated throughout the entire spleen. In the kidney section the convoluted tubules showed moderate cloudy swelling and fatty change. In the esophageal ulcerations, necrosis, bacterial masses, and lymphocytic exudate were noted.

Microscopic examination of the vertebral marrow showed a reduction in cellular elements, particularly in the myeloid series. There appeared to be a maturation arrest at the myelocyte level. Erythroblasts were present in normal numbers. In addition, it was of interest to note areas in which poorly stained marrow cells were mixed with fibrin, producing the appearance of focal necrosis in the bone marrow (see Figs. 1 and 2).

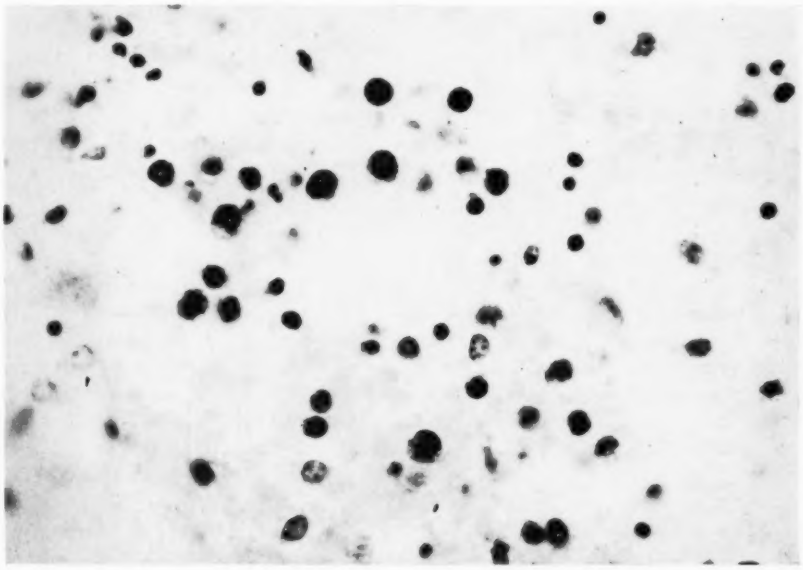


Fig. 2.—Vertebral bone marrow. Giemsa stain (×960). Erythroblasts, lymphocytes, reticulum cells, and occasional myelocytes are shown.

COMMENT

Although Case 3 was probably doomed by a fatal disease, the immediate cause of death was, no doubt, granulocytopenia, probably induced by sulfapyridine. More frequent blood counts during the period of treatment might have avoided this complication. The clinical aspects and autopsy findings with regard to the granulocytopenia were generally similar to those described in the patients treated with sulfanilamide. Sulfapyridine seems to have the same potential danger in the production of granulocytopenia as does sulfanilamide. Rather large doses over a period of two or three weeks may result in fatal granulocytopenia. Two side lights with regard to the endocarditis are of interest. One is the early improvement in the febrile state after the drug was started, although blood cultures remained positive. The other is the unusually small size of the vegetations. We have recently seen at autopsy in another patient who died of cardiac failure with subacute bacterial endocarditis, apparent reduction of vegetations to almost microscopic size after treatment with sulfapyridine.

FATAL HEMOLYTIC ANEMIA INDUCED BY SULFANILAMIDE

Hemolytic anemia is recognized as a relatively frequent complication of sulfanilamide therapy that develops after a few days of drug administration, and responds readily to treatment, particularly withdrawal of the drug and blood transfusion. Two fatal cases of hemolytic anemia are reported in the literature.[14, 15] The following is a report of another such case.

CASE 4.—(Los Angeles County Hospital, No. 247-892.) A 51-year-old white male, carpenter, was admitted to the Hospital on June 12, 1939, with a typical but not severe erysipelas, involving the external nares, of three days' duration. The past history was negative except for anginal attacks for three and one-half years. His temperature was 99.2° F., and he did not appear acutely ill. Urinalysis showed no albumin or sugar. Blood Wassermann was negative. His blood count on admission was hemoglobin 144 per cent (Sahli), erythrocytes 4.42 million, and leucocytes 14,900. Icterus index on June 13 was less than 10 units. During the next three days the patient received 25 Gm. of sulfanilamide, following which the blood level reached 7 mg. The following day deep jaundice was noted and the blood count now showed hemoglobin 42 per cent (Sahli), erythrocytes 1.75 million, and leucocytes 33,750, with 82 per cent polymorphonuclear leucocytes. The icterus index was 80 units. On this day the urine was noted to be very dark brown, with a positive benzidine test and no red blood cells. The urinary reaction was not recorded.

The clinical diagnosis was erysipelas, complicated by acute hemolytic anemia induced by sulfanilamide. Treatment consisted of repeated small transfusions of both whole and citrated blood. The patient belonged to group 0, and received a total of eleven transfusions between June 16 and June 22. A severe chill followed the first transfusion, but no reaction occurred after any of the others. During the first few days his general condition was poor, with dark urine, prominent icterus, and considerable lethargy. On June 20 the urine was clear, amber, and showed 150 to 200 red blood cells per high dry field in the centrifuged sediment. During the next week the patient's anemia improved and his jaundice diminished. There was a progressive increase in erythrocytes to 4.32 million on June 29, with 72 per cent hemoglobin. The leucocytes fluctuated considerably between outer limits of 33,000 and 9,800 per c. mm.

It thus appeared that the patient had recovered from his hemolytic anemia, but during the next week his condition grew worse, with two major manifestations. One was the occurrence of subcutaneous abscesses on the scalp and hands; the other was the development of progressive stupor following an epileptiform convulsion on June 28. His temperature, which had returned to normal during the first week, remained so, except for one rise to 101° F. His stupor deepened into coma and he died on July 11, 1939.

Autopsy was performed seventeen hours after death by Dr. Clara Margoles. Several subcutaneous abscesses, some draining thick pus (staphylococci in Gram stain smear) were still present. Small abscesses had also developed at the site of previous erysipelas of the nose. No icterus was noted. No lesions were found in the brain. Severe coronary sclerosis was present. The lungs showed patchy bronchopneumonia. The liver weighed 1,200 Gm.; cut surfaces were firm and dark brown. The spleen weighed 110 Gm.; cut surfaces were flabby but not soft, with pulp not unusually prominent. The kidneys together weighed 320 Gm.; capsules stripped readily from an unusually brick red-brown surface, which on cut surface showed similar pigmentation, particularly in the medullary portions. In addition, the parenchyma was softened, and a number of miliary abscesses were noted. The bone marrow of the vertebrae was brown, and that of the femur was fatty. The post-mortem blood creatinine was 5.0 mg. per 100 c.c.

The microscopic examination of the liver showed moderate cloudy swelling, slight fatty change, and with the Turnbull stain, large quantities of iron-containing pigment, often located in cells lining the sinusoids. In the spleen there was also a marked increase over normal in the amount of iron-containing pigment present, both intra- and extracellularly. The pulp was crowded with leucocytes, and there was some evidence of erythrophagocytosis.

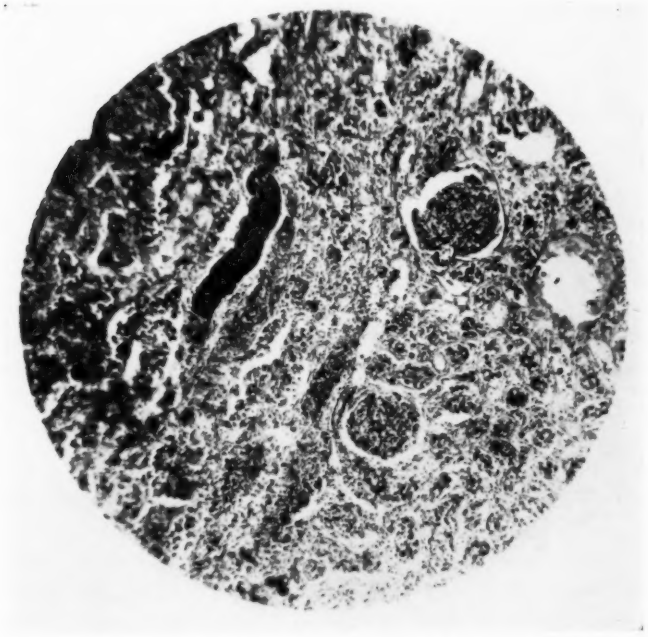


Fig. 3.—Kidney cortex (×100). Tubular degeneration and obstruction of lumen in cortex and medullary rays by hemoglobin casts.

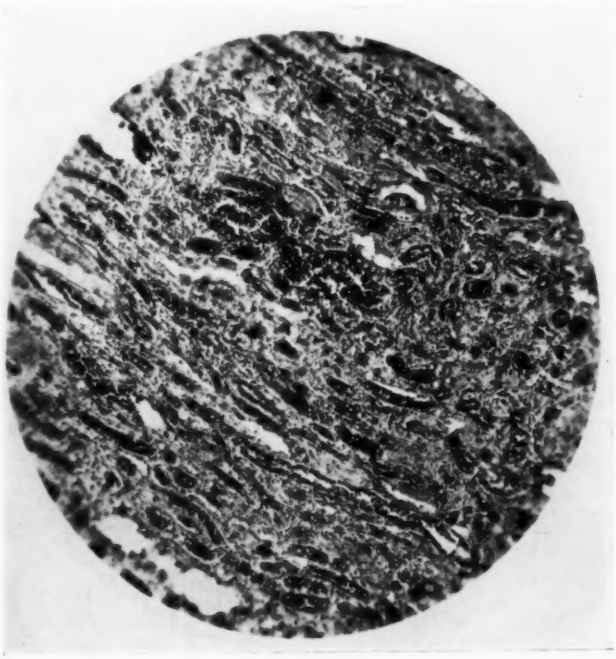


Fig. 4.—Kidney medulla (×100). Tubular degeneration and obstruction of lumen in cortex and medullary rays by hemoglobin casts.

The most striking changes were in the kidney. Here, in addition and apart from the miliary abscesses occasionally seen, there was marked tubular degeneration of the convoluted tubules and the loops of Henle. In the lumina of these tubules was disintegrated epithelium, as well as masses interpreted as blood pigment (see Figs. 3 and 4). Amorphous material was also noted in some of the collecting groups. A small amount of this material took the iron stain. Some of the epithelial casts showed partial calcification. There was no evidence of regeneration in the scattered tubules, which showed considerable epithelial necrosis. Glomeruli and blood vessels appeared essentially normal. Section of the vertebral marrow stained with hematoxylin and eosin showed a normal cellular distribution, except, perhaps, for some increase in nucleated blood cells.

COMMENT

The case herein reported conforms to the previous descriptions of the hemolytic anemia reaction. The anemia develops rapidly, within a few days after the administration of ordinary doses of the drug. In the two previously reported fatal cases death was attributed largely to the anemia, and nonspecific changes secondary to hemolysis and anemia were described at autopsy. In the present case, it is noted that the patient had recovered from the anemia, but died from uremia. From the changes in the kidney, there is reason to believe that the uremia occurred by obstruction and also direct damage to the kidney tubules by products of hemoglobin disintegration, e.g., acid hematin. Hemolytic anemia thus in this case caused death by delayed effects on the kidney. This effect is not specific for sulfanilamide, and has been described for other conditions, particularly after incompatible blood transfusions and in experimental hemoglobinemia. According to DeGowin, Warner, and Randall,[16] the renal damage is due not only to obstruction, but partly to direct injury to the tubules. In this connection, it is interesting to note that Wood's[14] patient was irrational, and vomited and bled from the gums before death; Koletsky's[15] patient died in coma. Some pigment deposit was noted in the kidney in the latter case.

Several important points need emphasis in regard to the treatment of the acute hemolytic anemia reaction. Blood transfusion is generally considered as indicated if the anemia becomes severe, as it usually does. It has been noted by Sharpe and Davis[17] that hemolytic reactions may complicate blood transfusion for hemolytic anemia, perhaps more than by mere coincidence. In the patient here reported a severe chill followed the first transfusion used. Additional hemolysis of the donors' corpuscles may have occurred at this time and added to the injury of the kidney produced by sulfanilamide hemolysis of the patient's corpuscles. A similar experience occurred in a case of hemolytic anemia and uremia recently reported,[18] where it was decided that the blood transfusion, rather than the sulfanilamide, was responsible. Attention has recently been called by Antopol and others[19] to another interesting phenomenon developing in the blood following sulfanilamide therapy, namely, the appearance of auto-agglutination together with hemolytic anemia. This property appeared when the blood was cooled and might be precipitated by giving blood transfusion of unwarmed citrated blood. Theoretically, there is potential danger in such a phenomenon to the kidney, by the plugging of glomeruli, as has been described in other instances of autohemagglutination, e.g., multiple myeloma.

The only way to avoid renal damage as a consequence of hemolytic anemia, is to alkalinize the urine in order to prevent the deposition of the obstructing

pigments in the renal tubules. It would appear that in severely ill patients likely to require both sulfanilamide and blood transfusions, or even in patients already showing signs of hemolytic anemia, rapid alkalinization of the urine should be carried out.

FATAL LIVER AND KIDNEY DAMAGE INDUCED BY SULFANILAMIDE

The probable causal relationship of the sulfonamide drugs to fatal granulocytopenia appears reasonably established by the case reports, and is in keeping with the chemical nature of these drugs, and with observations on their effect on the leucocyte count. Hemolytic anemia is also well established as a more frequent, but less dangerous, complication. Severe liver damage is a more recently described, and not as well established, toxic manifestation. Long, Bliss, and Feinstone[6] indicate that jaundice (without anemia) occurred in 0.6 per cent of 307 adult patients treated, and was controlled by stopping the use of sulfanilamide. Hageman and Blake[20] noted one case of hepatitis, possibly due to sulfanilamide in 114 treated patients. More frequently, jaundice has occurred incidental to the well-established hemolytic anemia reaction. However, there have been a number of recent reports indicating an apparent primary liver damage following the use of sulfanilamide.[21-27] The case reported by Fitzgibbon and Silver[22] was quite interesting. After receiving 44 Gm. of sulfanilamide for gonococcal urethritis, their patient had a generalized skin eruption, and was perhaps slightly jaundiced. Six weeks later, after taking only 1 Gm. of sulfanilamide for a recurrent urethral discharge, he developed dermatitis, this time associated with enlarged liver and severe jaundice followed by oliguria. The highest icterus index before recovery was 200, at which time the blood nonprotein nitrogen was 75 mg. per cent. Garvin's report[23] presented five cases in which the sulfanilamide appears to have been responsible for liver damage. The dosages of sulfanilamide employed were not excessive (maximum 50 Gm.) and were used in a conventional manner for diseases in themselves not serious. Jaundice was the prominent feature (highest icterus index noted was 160) and could not be explained on the basis of hemolytic anemia. It is interesting to again note the frequent association of dermatitis with the hepatitis. One of the patients died, but necropsy was not obtained. He also referred to two deaths with jaundice, commented on without detailed description in the report of Bannick and co-workers.[25] The week following Garvin's report, a case of acute yellow atrophy following sulfanilamide medication was reported by Cline[26] in the same journal. The patient was a white male of 18 years, who took an unknown quantity of sulfanilamide for gonorrheal urethritis, developed a clinical picture of hepatic insufficiency with an icterus index of 166, nonprotein nitrogen 33, and showed severe liver and kidney damage at necropsy. Greene and Hotz,[27] in their recent review of literature on the liver and biliary tract, mention four additional cases, with two deaths and one necropsy. Case 5 is a summary of the important clinical and necropsy data in an additional case of apparent liver and kidney damage following the use of sulfanilamide.

CASE 5.—(Los Angeles County Hospital, No. 607-856.) The patient was a Filipino male, aged 28 years, who entered the hospital because of pain, swelling, and dysfunction of the

left shoulder, knee, and hip joints. The referring physician stated that the patient was a periodic heavy drinker, but not in recent weeks. There was no known history of venereal disease or treatment, and no previous drug therapy for the present illness. Physical examination revealed a low-grade fever, evidence of acute polyarthritis, and purulent discharge from the urethra. Blood count showed hemoglobin 95 per cent (Sahli); leucocytes 11,000, with 80 per cent polymorphonuclear leucocytes. Gram-negative intracellular diplococci were found in the urethral discharge. The blood Wassermann and Kahn tests were positive. A small amount of serous fluid was aspirated from the left ankle joint, which contained a few leucocytes, but no bacteria on smear or culture.

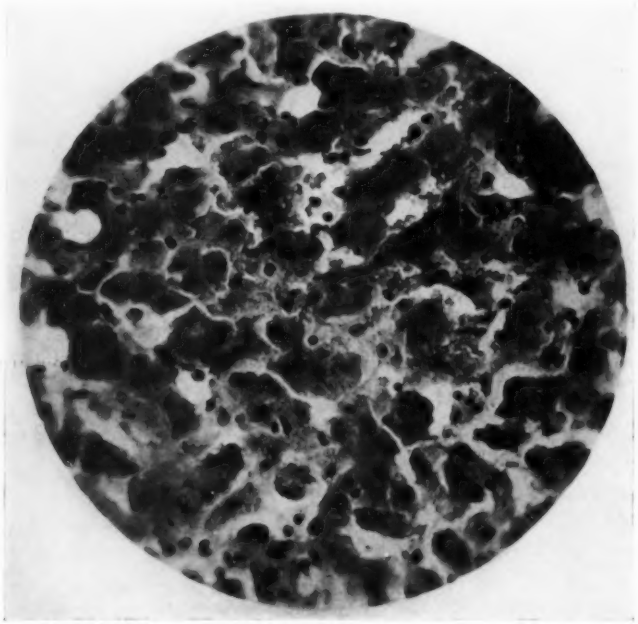


Fig. 5.—Liver, showing fragmented and swollen cell cords with cellular degeneration (×400).

The clinical diagnosis was gonococcal urethritis, with early polyarthritis, and the patient was started on sulfanilamide. Between May 6 and May 11, 1938, thirty-eight doses, totaling 34 Gm., were given. No antisyphilitic treatment was administered. On May 10 (one day before sulfanilamide was discontinued) jaundice was noted for the first time, but had probably previously been present and masked by the patient's native color, since blood drawn on that day had an icterus index of 220, and gave a prompt positive direct van den Bergh reaction with 29.1 mg. per cent of bilirubin. The urine contained albumin, bile, and a few hyaline casts. The patient's jaundice deepened, a maculopapular rash developed on May 14 (thought to look more like drug rash than secondary syphilis), and the patient became irrational. A blood transfusion was given without apparent improvement. The urethritis had meanwhile subsided. He developed Cheyne-Stokes respiration and died in coma.

At autopsy, jaundice and slight swelling of the left shoulder joint were still evident. No gross lesions were noted in the brain, heart, aorta, or gastrointestinal tract. The lungs were moderately hyperemic and edematous, but without gross consolidation. The liver weighed 2,300 Gm. Its external surface was smooth with tense capsule. Surfaces made by sectioning were softer than normal, with somewhat obscured lobular markings of mottled green to light brown. The gall bladder was of average size and thickness, but contained only a small quantity of inspissated bile. The biliary tract and portal system were normal. The spleen weighed 230 Gm., its pulp was prominent, dark purple, and somewhat softened. The kidneys together weighed 575 Gm. and were similar in appearance, each being about twice

normal size. The tense capsule stripped readily from a swollen, bile-stained surface. Surfaces made by sectioning were swollen, soft, with markings accentuated by bile staining. The urinary bladder contained a few drops of bile-stained urine. Prostate and seminal vesicles appeared grossly normal, as did the urethra throughout its extent. Small quantities of slightly bile-stained fluid were present in the right ankle and left shoulder joints, the synovia of the latter being slightly thickened. The post-mortem blood nonprotein nitrogen was 142, creatinine 14.4, and urea nitrogen 85 mg. per cent. No sulfanilamide was detected in the post-mortem blood; 2 mg. per cent were found in the liver (Table I).

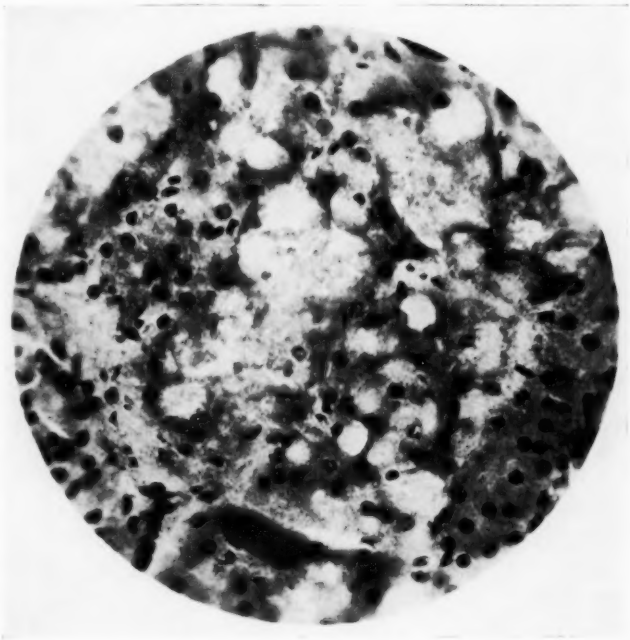


Fig. 6.—Kidney, showing focal necrosis of convoluted tubules (×400).

The histologic findings of interest were chiefly in the liver and kidneys. In the liver there was considerable disorganization of cell cords, marked cloudy swelling, some fatty change, and degenerative changes in the nuclei, with only a few scattered zones of actual necrosis (see Fig. 5). Numerous lymphocytes and plasma cells occurred in the periportal areas, but no connective tissue proliferation was noted. Considerable bile pigmentation was present. The kidneys showed fairly normal glomeruli, with both hematoxylin-eosin and azocarmine stains. Marked tubular degeneration was evident, with many areas in which groups of convoluted tubules were completely necrotic (see Fig. 6). Masses of bile pigment were scattered throughout the tubules. Very little iron pigment was shown in the Prussian blue stain. In the spleen there was a moderate increase of iron pigmentation. Bone marrow smears and sections were not remarkable. No organisms were seen in smears from the spleen, and cultures were sterile.

COMMENT

The histologic appearance of the liver is not that of acute yellow atrophy. It does show evidence of hepatic degeneration, but not as much as would be expected from the degree of impaired liver function demonstrated clinically. There is, however, no reason for believing, from either clinical or laboratory evidence, that hemolytic anemia was responsible for any appreciable part of

TABLE I

AUTOPSY NO.	CLINICAL DIAGNOSIS	CAUSE OF DEATH	TOTAL DOSAGE OF SULFANILAMIDE	DATES ADMINISTERED	DATE OF DEATH	SULFANILAMIDE IN BLOOD* MG./PER CENT	SULFANILAMIDE IN TISSUES† MG./PER CENT
21064	Pyelonephritis	Uremia	27 Gm.	Dec. 4-12 (1938)	Dec. 12 (1938)	---	8.4 liver
20105	Influenzal meningitis	Same	10.5 Gm.	June 8-11	June 12	---	12.0 liver
20927	Scarlet fever	Granulocytopenia	101 Gm.	Nov. 2-18	Nov. 25	5.5 (Nov. 17)	2.5 liver
20440	Puerperal sepsis	Streptococcus viridans septicemia	46 Gm.	July 8–Aug. 10	Aug. 18	---	0 liver
19798	Thrombophlebitis erysipelas		24 Gm.	Mar. 24–April 1	April 13	---	0 liver
21055	Pneumococcal meningitis	Same	730 Gm.	Oct. 2–Dec. 4	Dec. 16	33 (Nov. 1) 15 (Dec. 12)	3.1 liver 2.7 kidney
19973	Gonorrheal arthritis	Hepatorenal syndrome	34 Gm.	May 6-11	May 16	0 (Postmortem blood)	2 liver
DA 724	Streptococcus viridans subacute bacterial endocarditis	Same	24‡ Gm.	Jan. 10-15 (1939)	Jan. 16	---	3.9 liver 2.3 spleen 8.3 kidney

*Determinations were by the method of Marshall for free sulfanilamide, and the value given is that last determined.

†Five grams of tissue were macerated with 10 c.c. of water and mixed with 5 c.c. of trichloracetic acid. The free sulfanilamide content of the filtrate was determined.

‡This patient received sulfapyridine. The usual sulfanilamide standards were used in the determinations.

the patient's jaundice. The lesion in the kidney is more striking than that of the liver, and is in keeping with the patient's picture of progressive oliguria and failing kidney function. It suggests direct injury to the kidney, largely tubular, probably by the same agent that injured the liver, although it might conceivably be included in the category of "hepatorenal syndrome," with kidney damage dependent on the products of liver damage.

As in the previously reported cases, the cause of the injury to the liver and kidneys is not clear. The drug was administered to a patient not seriously ill, in an amount frequently used in clinical practice. Although sulfanilamide is acetylated by the liver and excreted by the kidney, neither the free nor conjugated form has been shown to be injurious to these organs by experimental methods. Hageman[7] reported no apparent damage to the liver and kidneys, and noted only excess deposits of iron-containing pigment in the spleen, after the administration of lethal doses to mice. It has, however, been noted that the bromsulphthalein excretory function of the liver is impaired in patients re-

ceiving sulfanilamide. It is also possible that chronic alcoholism may have predisposed the patient to hepatic damage.

Although the mechanism of liver and kidney damage is not understood, it is clear from experimental observation, that with conjugation impaired by liver damage and excretion impaired by kidney damage, retention of free sulfanilamide might occur in the tissues. It is also theoretically possible that the retention of the drug in a particular organ need not necessarily be reflected in the blood sulfanilamide level. We, therefore, thought it of interest to determine the tissue sulfanilamide content at autopsy. Since few figures are available on human tissues, control determinations were done on tissues from several other autopsies where sulfanilamide had been administered prior to death. The control observations are not very satisfactory because of the variation in amount of drug given, and in the period elapsing from cessation of administration to death. The results are given in Table I.

Marshall and collaborators[28] have shown that under normal conditions in experimental animals, free sulfanilamide is rather evenly distributed throughout most body tissues. Engelfried[29] recorded similar findings in human autopsy material from one patient. Greene and Hotz[27] noted considerable increase in the concentration of sulfanilamide in the liver as compared to the lung in a case of hepatic degeneration, apparently due to sulfanilamide.

Although only the liver was analyzed in our case (19973), it does not show an abnormally high value as compared with the findings in other cases at a similar period after administration of the drug. Further chemical studies of tissue at autopsy, especially where there have been toxic effects, may be of interest.

SUMMARY

Five deaths are reported following the administration of sulfonamide drugs, three from granulocytopenia, one from hemolytic anemia, and one from liver and kidney damage. In a patient receiving 101 Gm. of sulfanilamide for scarlet fever, and in another patient receiving 64 Gm. of sulfanilamide for streptococcal sore throat, fatal granulocytopenia developed. A third case of fatal granulocytopenia occurred in a patient who was given 88.5 Gm. of sulfapyridine in the treatment of bacterial endocarditis. In two of these patients the bone marrow was examined at autopsy and showed maturation arrest of the myeloid series at the myelocyte level. A typical acute hemolytic anemia developed within three days in a patient treated for erysipelas with 25 Gm. of sulfanilamide. Interference with renal tubular function by precipitated hemoglobin derivatives was thought to be a major factor leading to death in this patient. The fifth case showed clinical evidence of severe liver and kidney damage developing after the administration of 34 Gm. of sulfanilamide for gonorrheal arthritis. Degeneration of liver cells and necrosis of renal tubular epithelium were found at autopsy.

REFERENCES

1. Sheckett, H. A., and Price, A. E.: Fatal Granulocytopenia Following Administration of Sulfanilamide, J. A. M. A. **112**: 823, 1939.
2. Ottenberg, R.: Clinical Experiences With Sulfanilamide Therapy, New York State J. Med. **39**: 418, 1939.

3. Corr, Philip, and Root, Ralph N.: Death From Granulocytopenia After Sulfanilamide Therapy, J. A. M. A. **112**: 1939, 1939.
4. Sailer, S.: Subacute Bacterial Endocarditis Treated With Sulfanilamide Resulting in Granulocytopenia and Death, Am. J. Path. **9**: 269, 1939.
5. Garvin, Curtis: Complications Following the Administration of Sulfanilamide, J. A. M. A. **113**: 288, 1939.
6. Long, Perrin H., Bliss, Eleanor A., and Feinstone, W. H.: Mode of Action, Clinical Use and Toxic Manifestations of Sulfanilamide, J. A. M. A. **112**: 115, 1939.
7. Hageman, P. O.: Toxicity of Sulfanilamide: A Study of the Pathological Lesions in White Mice, Proc. Soc. Exper. Biol. & Med. **37**: 119, 1937.
8. Long, Perrin H.: Sulfapyridine, J. A. M. A. **112**: 538, 1939.
9. Johnston, F. D.: Agranulocytosis Following Treatment With M & B 693, Lancet **2**: 1200, 1938.
10. Coxon, R. V., and Forbes, J. R.: Agranulocytic Angina Following Administration of M & B 693, Lancet **2**: 1412, 1938.
11. Barnett, H. L., Hartmann, A. F., Perley, A. M., and Ruhoff, M. B.: The Treatment of Pneumococcic Infections in Infants and Children With Sulfapyridine, J. A. M. A. **112**: 527, 1939.
12. Rosenthal, Nathan, and Vogel, Peter: Granulocytopenia Caused by Sulfapyridine in Children, J. A. M. A. **113**: 584, 1939.
13. Dolgopol, Vera B., and Hobart, Harold M.: Granulocytopenia in Sulfapyridine Therapy, J. A. M. A. **113**: 1012, 1939.
14. Wood, Harold: A Fatality From Acute Hemolytic Anemia Which Developed During the Administration of Sulfanilamide, South. M. J. **31**: 646, 1938.
15. Koletsky, Simon: Fatal Hemolytic Anemia Following the Administration of Sulfanilamide, J. A. M. A. **113**: 291, 1939.
16. DeGowin, E. L., Warner, E. D., and Randall, W. L.: Renal Insufficiency From Blood Transfusion, Arch. Int. Med. **61**: 609, 1938.
17. Sharpe, J. C., and Davis, H. H.: Severe Reactions Following Transfusions in Hemolytic Jaundice, J. A. M. A. **110**: 2052, 1938.
18. Cabot Case No. 25221: New Eng. J. Med. **220**: 924, 1939.
19. Antopol, William, Applebaum, Irving, and Goldman, Lester: Two Cases of Acute Hemolytic Anemia With Auto-agglutination Following Sulfanilamide Therapy, J. A. M. A. **113**: 488, 1939.
20. Hageman, P. O., and Blake, F. G.: Clinical Experience With Sulphanilamide in the Treatment of Beta Hemolytic Streptococcic Infections, Am. J. M. Sc. **195**: 163, 1938.
21. Saphurstein, H.: Hepatitis and Toxic Erythema With Desquamation Due to Sulfanilamide, Urol. & Cutan. Rev. **42**: 101, 1938.
22. Fitzgibbon, Paul, and Silver, Barney: Toxic Necrosis of Liver Following Use of Sulfanilamide, California & West. Med. **50**: 123, 1939.
23. Garvin, Curtis F.: Toxic Hepatitis Due to Sulfanilamide, J. A. M. A. **111**: 2283, 1938.
24. Gertler, W.: Injury to Hepatic Parenchyma With Aspect of Acute Yellow Atrophy Following Prontosil Therapy of Recidivating Erysipelas, Dermat. Wchnschr. **106**: 725, 1938.
25. Bannick, Edward G., Brown, Alex E., and Foster, Frank P.: Therapeutic Effectiveness and Toxicity of Sulfanilamide and Several Related Compounds, J. A. M. A. **111**: 770, 1938.
26. Cline, E. W.: Acute Yellow Atrophy Following Sulfanilamide Medication, J. A. M. A. **111**: 2384, 1938.
27. Greene, Carl H., and Hotz, Richard: Liver and Biliary Tract (Review), Arch. Int. Med. **63**: 778, 1939.
28. Marshall, E. K., Jr., Emerson, Kendall, Jr., and Cutting, W. C.: The Distribution of Sulfanilamide in the Organism, J. Pharmacol. & Exper. Therap. **61**: 196, 1937.
29. Engelfried, John J.: Further Observations on the Distribution and Concentration of Sulfanilamide in the Tissues of the Body After Enteral and Parenteral Administration, J. Michigan M. Soc. **38**: 129, 1939.

NUTRITION AND NERVOUS EXCITABILITY*

LOUIS MARKS AND H. NECHELES
CHICAGO, ILL.

IN STUDIES on basal metabolic rate of Chinese it was found that the relatively lower heat production of Chinese was probably due to their greater degree of relaxation. This conclusion was arrived at in the following way: It was assumed that since man's voluntary musculature is more or less relaxed when sleeping, one's basal metabolic rate should be equal during sleep, but different while awake, depending upon the individual's degree of muscular relaxation. To test this, the oxygen consumption of Chinese and Westerners was determined during both conditions. It was found that in most Chinese the basal metabolic rate did not change appreciably after they had fallen asleep, but that it dropped considerably in Westerners, with a relatively higher waking basal metabolic rate. Westerners with a low waking basal metabolic rate did not show such a drop while asleep (Necheles, 1928; Necheles, 1930; and Necheles and Loo, 1932).

It is believed that the Chinese consume less protein and protein of lower biologic value than the Westerner; this may explain indirectly their lower basal metabolic rate. It is well known, of course, that the specific dynamic action of proteins lasts only as long as their digestion products are absorbed from the intestines and that, therefore, the simple concept of a higher or lower specific dynamic action would not be sufficient to explain a higher or lower basal metabolic rate in the completely fasting condition. It was thought possible, however, that a diet high in proteins might lower the threshold of muscular as well as of nervous and of reflex excitability beyond the period of digestive absorption, thereby raising muscle tonus and number or extent of reflex movements, and consequently oxygen consumption. With this idea in view, experiments were performed by one of us (H. N.), together with Dr. Ernest DeVries (Department of Neurology, at the Peiping Union Medical College, China. Chinese subjects were employed, and the threshold excitability of the branch of the facial nerve supplying the risorius muscle, as well as the ulnar nerve in the elbow, and movement of the fifth finger were recorded. A clinical wall plate was employed, and the amount of current used was read in milliamperes. The indifferent electrode was held against the chest, while the different one was applied by hand to the marked nerve point. Both electrodes were soaked in saline solution. The kathodal closing contraction (K.C.C.), the anodal closure contraction (A.C.C.), and the kathodal opening contraction (K.O.C.) were determined. After a constant level of excita-

*From the Department of Gastrointestinal Research, Michael Reese Hospital, and the Department of Physiology, the University of Chicago.
Aided by the Rosalinda Klein Fund.
Received for publication, October 7, 1939.

bility had been established in fasting subjects, they were given, on different days, meals consisting of ½ lb. of broiled lean meat, or 120 Gm. of butter on a slice of toast, or a carbohydrate meal consisting of a fruit pudding with sugar and glutinous rice. After the meal the nervous thresholds were determined in twenty-minute intervals, and the oxygen consumption and the carbon dioxide production were measured. The results were quite uniform and indicated that proportional to the rise of the specific dynamic action after a meat meal (but not as regularly or extensively after fat, carbohydrate, or fasting), the nervous thresholds would drop considerably and return to control values at about the same time as the oxygen consumption and the carbon dioxide production.

Since this phenomenon might have been caused by a variation in skin resistance following ingestion of meat, control experiments were performed in which, following the technique of Richter (1926), the epidermis above the nerve point on one side of the body was perforated with a surgical needle, and the different electrode applied to that spot; the contralateral side with nonperforated skin served as control. It was found that following this procedure the lowering of nerve thresholds following a protein meal was no longer detectable. This proved, indirectly, that the results obtained in the previous experiments had been due to changes in skin resistance rather than to variations in nervous thresholds. At this point the experiments had to be interrupted because one of us (H. N.) left China. They were taken up again in Chicago on white persons. The technique was basically the same, except that small zinc electrodes were fastened to the skin with collodion and adhesive, that Cambridge electrode jelly was used instead of saline moistened pads, and that a more sensitive amperemeter, a more accurate rheostat, and radio batteries as source of current were employed. To our surprise, results were entirely different from those found in Peiping. Seven male and 6 female persons were employed. It was found that the nervous threshold, when followed over prolonged control periods of fasting, showed rhythmic variations, and that after carbohydrate, fat, or protein meals no significant or constant variation in nervous threshold was found either on the unperforated or on the perforated skin.

DISCUSSION

The observations in China were made by two independent observers (Dr. DeVries and H. N.), both of whom obtained essentially the same results. The tests in Chicago were performed by one of us (L. M.) after having obtained proficiency in the technique used, and repeating each reading at least three times. We offer the following possible explanations of the discrepancy between our results on Chinese in Peiping and on white persons in Chicago: in case of the subjects in Peiping an unaccustomed high protein meal may have produced a change in skin resistance. Unfortunately, we were not able to obtain Chinese subjects in Chicago, although results might not be the same as in China because we know that the nutritional standards of the American Chinese are quite different from those in their homeland. The second possible explanation may be found in the fact that in the experiments done in Peiping,

the different electrode may not have been applied to the same spot at each test; this is not probable, however, in view of similar curves in a great number of tests done by two different observers. A third possible explanation may be seen in the difference in climate between Peiping and Chicago, and its effect on skin resistance.

A number of other experiments were performed in Chicago in order to analyze the relation between food substances and reflexes under more controlled conditions. In consequence of the negative results obtained, they will be mentioned only briefly:

1. Reflex time (Tuerck) in frogs before and after injection of, or perfusion with, amino acids in buffered solutions.

2. Height and reaction time of knee jerk and chronaxie of ipsilateral flexor and crossed extensor reflexes in anesthetized dogs before and after intravenous injection of amino acids.

3. Reaction time in man (Dunlop chronoscope) before and after carbohydrate, fat, or protein meals.

4. Height of knee jerk (mechanical hammer) in man before and after above meals, as well as before and after ingestion of amino acids.

SUMMARY

In order to explain the difference in basal metabolic rate between Chinese and Westerners, experiments were conducted in China and in the United States to determine the effect of various foods on the neuromuscular system. It was found that the skin resistance of Chinese in Peiping apparently decreased after a protein meal, while that of Westerners in Chicago did not.

REFERENCES

Necheles, H.: Data and Observations on Several Factors Influencing Basal Metabolism in China, Chinese J. Physiol. **1**: 80, 1928.

Necheles, H.: Basal Metabolism in Orientals, Am. J. Physiol. **91**: 2, 1930.

Necheles, H., and Loo, C. T.: Über den Stoffwechsel der Chinesen, Chinese J. Physiol. **6**: 129, 1932.

Richter, C. P.: New Methods of Obtaining Electromyogram and Electrocardiogram From Intact Body, J. A. M. A. **87**: 1300, 1926.

THE INTRAVENOUS ADMINISTRATION OF SUCROSE SOLUTIONS AS A MEANS OF PRODUCING INTENSE DIURESIS*

HENRY F. HELMHOLZ, M.D., AND JESSE L. BOLLMAN, M.D.
ROCHESTER, MINN.

IN THE acute stage of urinary infections the washing out of the urinary passages is one of the most effective means of treatment. The use of large amounts of fluid by mouth or by other routes is often sufficient to bring about a flow of urine. In some cases, however, this alone is not sufficient and a more powerful diuretic is necessary. It is our object to find out what solution can be given with a minimum of harm to the tissues and yet produce a maximal diuresis.

From former studies on the excretion of bacteria after the injection of 20 per cent sucrose solution, it was shown that a sufficiently intense diuresis could be produced so that one-half of the cultures of 0.5 c.c. of urine were sterile, whereas before the diuresis was started the urine in the same quantities contained innumerable bacteria.[1] This observation led us to investigate the value of sucrose as a diuretic and to find out its toxicity and its relative value as compared with dextrose, sorbitol, urea, and sodium sulfate.

For a comparison of dextrose and sucrose as safe diuretic agents, there were available experiments carried out some years ago on rabbits. Thirteen rabbits were injected with 20 per cent solution of dextrose, varying in amounts from 60 to 108 c.c. per kilogram of body weight per hour, and 16 animals were injected with 20 per cent solution of sucrose, varying in amounts from 107 to 167 c.c. per kilogram per hour. The average volume of urinary output of the 13 animals receiving dextrose was 73 per cent of the volume injected, and for the 16 animals receiving sucrose the output was 94 per cent. This would indicate that even though the tonicity of the dextrose solution was practically twice that of the sucrose, its effect was 21 per cent less. When the quantities injected are taken into account, this difference is very much greater because much larger amounts of sucrose were injected, as is seen in Table I. The greater danger of these injections of dextrose, although much smaller in amount than the sucrose injections, is seen from the death of 92 per cent of the animals that received dextrose in contrast to the death of 25 per cent of the animals that received a 65 per cent greater volume of 20 per cent sucrose solution. Thus it is evident that sucrose solution is both better and safer than dextrose solution for purposes of diuresis when large amounts are to be injected in a short period (Table I).

In our experiments with 20 per cent solution of sucrose we found that the maximum diuresis in one hour was obtained without subsequent mortality when 100 c.c. for each kilogram of body weight was injected in one hour. Compari-

*From the Mayo Clinic, Rochester.
Received for publication, December 20, 1939.

sons were made of the diuretic and lethal effect of equal volumes (100 c.c. per kilogram) of solutions of equal osmotic pressure of sodium sulfate, sorbitol, and urea, the latter dissolved in physiologic salt solution.

TABLE I

A COMPARISON BETWEEN THE TOXICITY OF A SINGLE LARGE INJECTION OF SUCROSE AND A SINGLE LARGE INJECTION OF DEXTROSE

	16 ANIMALS INJECTED WITH 20% SUCROSE Average Injection 140 c.c. per kg. in 1 Hour	13 ANIMALS INJECTED WITH 20% DEXTROSE Average Injection 85 c.c. per kg. in 1 Hour
Died within 1 hour	6.2%	69.2%
Died within 24 hours	25.0%	92.3%
Recovered	75.0%	7.7%

The Diuretic Effect of 2.8 Per Cent Solution of Sodium Sulfate When Administered Intravenously in a Dose of 100 c.c. per Kilogram per Hour.—The animals did not tolerate the solution well, so that injections were given only three times a week rather than five times a week as were the sucrose injections. The urinary output for every 100 c.c. injected was 85 c.c. for a total of eighty-three injections in three animals. The two animals that received thirty-two and thirty-four injections, respectively, died.* The one animal that received only seventeen injections recovered. The phenolsulfonphthalein output ranged in the animals between 57 and 100 per cent. The changes in the kidneys of the two animals receiving the large number of injections were relatively mild as compared with those in similar animals receiving sucrose. The tubular epithelium was slightly swollen and vacuolated, but otherwise there were no changes in the kidneys.

The Diuretic Effect of 3.5 Per Cent Urea Solution in Normal Saline When Administered Intravenously in a Dose of 100 c.c. per Kilogram per Hour for One Hour.—The administration of urea in water produced hemoglobinuria, so that urea was administered in normal saline solution. This solution was well tolerated. Six animals were injected, one animal receiving sixteen injections, another receiving twenty-six injections, and the rest a smaller number of injections. These animals received three injections a week. The diuresis was 73 c.c. per hour for each 100 c.c. per kilogram per hour injected. The animal receiving the twenty-six injections was killed twenty-four hours after its last injection. At necropsy the kidneys were not enlarged and appeared normal on section. Microscopically the convoluted tubular epithelium was swollen, the protoplasm granular and full of vacuoles. Many tubules were filled with granular debris. Some tubules were lined with narrow cells which had a shredded border toward the lumen. The findings resembled somewhat those found on injections of sucrose, except that the swelling of the cells was not so great.

The Diuretic Effect of 11.7 Per Cent Solution of Sorbitol When Administered Intravenously in a Dosage of 100 c.c. Per Kilogram Per Hour for One Hour.—Recently suggested for its dehydrating effect, it was thought that sorbitol might have a diuretic effect superior to that of sucrose. Single experi-

*Four other rabbits died within eighteen hours after receiving one, one, two, and four injections, respectively.

ments on two rabbits showed in one case an excretion of 80 c.c. and in the other 90 c.c. of urine for each 100 c.c. injected.

TABLE II

AVERAGE OUTPUT IN ONE HOUR DURING INJECTIONS OF 100 C.C. PER KG. PER HOUR OF ISOTONIC SOLUTIONS OF SODIUM SULFATE, UREA, AND SUCROSE

NATURE OF SOLUTION INJECTED	URINARY OUTPUT, C.C. PER KG.	NUMBER OF INJECTIONS
2.8% solution of sodium sulfate	85	83
3.5% solution of urea	73	42
20% solution of sucrose	131	33

EFFECT OF INJECTIONS OF SUCROSE

The comparison of the effectiveness of sodium sulfate, urea, and sucrose as diuretics is seen in Table II. It will be noted that in the special series of thirty-three injections of sucrose, 131 c.c. of urine per kilogram were returned within the hour when 100 c.c. of 20 per cent solution of sucrose per kilogram had been injected intravenously. This was the only one of the substances in which more was returned in the hour than was injected. We found the sucrose to be safer than the sodium sulfate which gave the next best results. It is also of interest to compare the percentage of excretion of urine when 100, 75, and 50 c.c. of 20 per cent solution of sucrose for each kilogram of body weight was injected within an hour. The figures were very close—131, 136, and 136 per cent of the amounts injected. It would seem that a 20 per cent solution of sucrose was the most effective, and under these circumstances the safest, as well as a very inexpensive, diuretic solution for intravenous use.

Early in our experiments it was evident that the intravenous injection of 20 per cent solution of sucrose produced extreme hydropic degeneration of the convoluted tubules, and when large amounts were injected, it produced a lowering of the phenolsulfonphthalein output and subsequently a urinary output that fell to zero. It was necessary, therefore, to determine whether these changes in the convoluted tubules were of a permanent or of a transient character and whether functionally the kidney showed permanent damage which would contraindicate the use of sucrose. In this connection we considered the possibility of functional incapacity of the convoluted tubules that might shed some light on their secretory activity, particularly with regard to reabsorption of water from the glomerular filtrate. Uniformly, however, the concentration of the urine following the injection indicated that in spite of the marked hydropic degeneration of the tubular epithelium this function remained intact. We, therefore, gave a number of animals repeated injections of 20 per cent solution of sucrose.

Of the animals injected we want to mention only three that received the greatest number of injections, one receiving thirty-eight, a second receiving fifty-two, and a third which received sixty-eight injections of 20 per cent sucrose in distilled water in one-hour periods. The essential data concerning these animals are given in Table III. The animals remained in good condition throughout the course of the experiments; rabbit 2 gave birth to six young twenty-five days after the last injection of sucrose. Attention is called to the maintenance

of good diuretic response to the injection of sucrose and also the phenolsulfonphthalein excretion which was not greatly impaired by the repeated injections.

TABLE III

EFFECT OF REPEATED INJECTIONS OF 20 PER CENT SOLUTION OF SUCROSE ON THE FUNCTION OF THE KIDNEY

NUMBER OF INJECTIONS	AMOUNT INTRAVENOUSLY INJECTED (C.C.) PER KILOGRAM PER HOUR	URINARY OUTPUT (C.C.) PER KILOGRAM PER HOUR	PERCENTAGE EXCRETION PHENOLSULFONPHTHALEIN
Rabbit 1, weight 2.3 kg., receiving 38 injections in 82 days.			
4	50	46	56
2	75	105	63
1. Left nephrectomy 3 days after 6th injection. Injections resumed after 23 days.			
12	50	42	44
11	75	66	40
9	100	63	48
2. Biopsy of right kidney 5 days after 38th injection.			
Rabbit 2, weight 2.4 kg., receiving 52 injections in 71 days.			
1	100	132	66
12	50	76	52
2	75	126	33
22	100	118	54
1. Left nephrectomy 1 day after 37th injection. Injections resumed after 5 days.			
15	50	56	44
2. Biopsy of right kidney 4, 39, and 105 days after 52nd injection.			
Rabbit 3, weight 2.0 kg., receiving 68 injections in 110 days.			
4	50	35	62
8	75	91	54
9	100	98	35
1. Left nephrectomy 3 days after 21st injection. Injections resumed after 8 days.			
10	50	46	34
10	75	57	30
12	100	59	25
2. Biopsy of right kidney 1 day after 53rd injection. Injections resumed after 5 days.			
15	50	49	28
3. Biopsy of right kidney 39 and 105 days after 68th injection.			

The kidneys and sections removed one to five days after several injections of sucrose appeared swollen with the cortex thickened. The convoluted tubules were greatly swollen; the epithelium of the convoluted tubules was large and clear, so that the cells resembled those of hypernephroma. The nuclei were small and pyknotic, and many cells did not appear to have nuclei. The glomeruli and the other structures of the kidney appeared to be normal. Sections removed one to five days after the last injection appeared similar, except that in the latter an increased staining of some regions of the convoluted tubules suggested an increase in the protoplasm of the swollen cells.

Fig. 1 illustrates the condition of the convoluted epithelium one day after the fifty-third injection of sucrose. Very few cell outlines could be made out. In portions of tubules only small shreds of protoplasm were seen and to a considerable extent the cells lining the tubules did not show nuclei. When cell out-

lines were distinguishable, the tubules were practically swollen shut. In some regions where cell outlines were represented only by small masses of granular material, the lumen of the tubules seemed large. The glomerular capsules were distended, so that there was much space about the glomerulus.

Sections taken thirty-nine days after the sixty-eighth injection indicated almost complete recovery from what seemed, in the sections taken within a few days after injection, to be irreparable damage to the convoluted tubules. The sections appear like those from the normal kidney of the rabbit, except perhaps that the cells of the convoluted tubules were slightly larger (Fig. 2). Sections taken one hundred and five days after the last injection were entirely indistinguishable from sections of normal kidneys.

Injection for Six Hours.—Having convinced ourselves that sucrose solution even in 20 per cent concentration injected five times a week for a number of weeks does not result in any permanent damage to the kidney and does not seriously interfere with its function, it next seemed indicated to continue the set rate of injection of 100 c.c. per kilogram per hour for longer periods. In extending the time, we are putting a much greater strain on the circulation as well as the kidney, and it is essential, in order to be successful, that there be a balance between intake and outgo. Former experiments had shown us that even when 50 per cent sucrose was injected, the kidney could not put out a concentration of sucrose of more than 10 per cent. As a result of a one-hour experiment we were pretty thoroughly convinced that sucrose is the best diuretic for intravenous use, but we felt that a few six-hour experiments with the various diuretics used in the one-hour experiments would be of confirmatory value.

Our first injections with 8 and 10 per cent sucrose solution in water showed us that the good diuresis of the early hours rapidly disappeared with a diminishing output of sodium chloride. The chloride concentration, as well as the volume of urine, was greatly reduced, and there was a retention of sucrose proportional to the water retention. Two of the three animals died, and the output for the six-hour period was only 66, 44, and 39 per cent of the amount injected.

To correct this lack of sodium chloride, as well as a lack of potassium and calcium, one-half strength Ringer's solution was used because it is approximately equivalent to the amount of inorganic salts excreted in the first hour of marked diuresis with sucrose in water. Using 8 per cent sucrose in one-half strength Ringer's solution, there was a surprising improvement in the output of urine—an average output of 88 per cent of the volume injected—and there was no definite retention of water, sucrose, or sodium chloride. The general condition of these animals at the end of the injection was practically normal. Not one of the eight injected animals died. One animal had two additional six-hour injections in the course of two months, excreting 92 per cent and 95 per cent of the amount injected the second and third times, and one animal had two injections in a period of a month.

Ringer's solution was injected at the same rate of 100 c.c. per kilogram per hour for six hours in five animals, one of which died during the course of the fifth hour. In all there was a marked retention of water and salt.

Three animals receiving 1.44 per cent urea in one-half strength Ringer's solution died within forty-eight hours, one dying after two and one-half hours

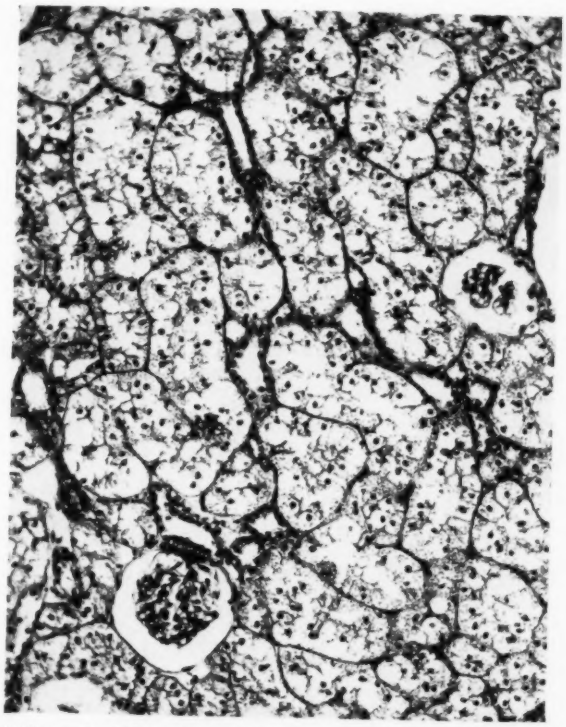


Fig. 1.—Section of kidney (×140) removed from rabbit 3 twenty-four hours after the fifty-third injection of 20 per cent sucrose.

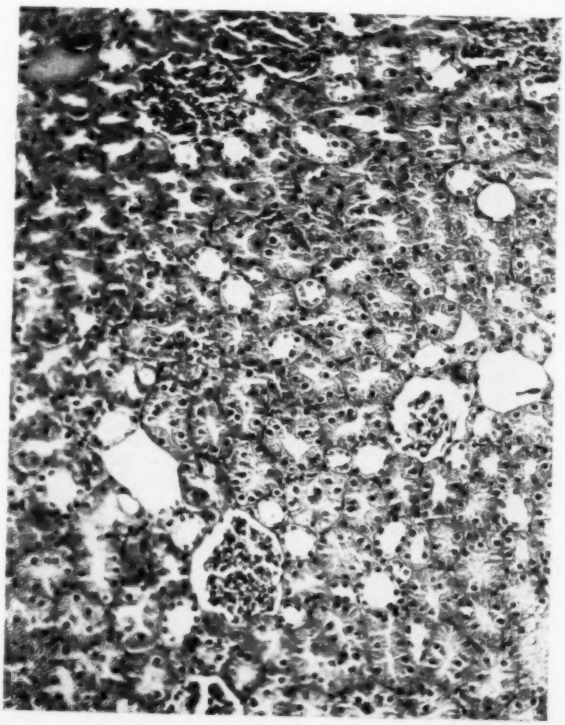


Fig. 2.—Section of kidney (×140) removed from rabbit 3 thirty-nine days after the sixty-eighth injection of 20 per cent sucrose.

of injection. Each showed a marked retention of water, urea, and sodium chloride.

Three animals received 1.55 per cent sodium sulfate in one-half strength Ringer's solution; one died four hours after the injection, and one within forty-eight hours. Each animal showed retention of water, sulfate, and sodium chloride during the injection period.

One of the four animals receiving 4.2 per cent dextrose in one-half strength Ringer's solution died at the end of four hours, and all showed retention of water and sodium chloride.

The two animals receiving 4.5 per cent sorbitol in one-half strength Ringer's solution lived and excreted 48 per cent of the volume injected, retaining sodium chloride proportional to the water retained. Five animals received 6 per cent sorbitol in the same solution and put out on an average 80 per cent, and three of the animals died within forty-eight hours.

TABLE IV

DIURESIS AFTER INJECTION OF 100 C.C. PER KILOGRAM PER HOUR FOR SIX HOURS

SOLUTION	PERCENTAGE OF INJECTED SOLUTION EXCRETED	NUMBER OF ANIMALS INJECTED	NUMBER OF ANIMALS WHICH DIED WITHIN 48 HOURS
1.44% urea in ½ Ringer's solution	38	3	3
Ringer's solution	47	5	1
4.5% sorbitol in ½ Ringer's solution	48	2	0
4.2% dextrose in ½ Ringer's solution	62	4	1
1.55% sodium sulfate in ½ Ringer's solution	73	3	2
6% sorbitol in ½ Ringer's solution	80	5	3
8% sucrose in ½ Ringer's solution	88	8	0

From Table IV it is to be seen that an 8 per cent sucrose solution in one-half strength Ringer's solution produces a fair balance of fluid, sodium chloride, and sucrose. As was to be anticipated the changes in the kidneys after a twenty-four-hour and a forty-eight-hour interval resemble very closely the changes in those experiments in which a 20 per cent sucrose solution was injected for one hour and the animal killed after twenty-four and forty-eight hours. These changes, however, did not interfere with the function of the kidney sufficiently to prevent an animal tolerating three such injections and at the last injection putting out a greater volume than in the previous one.

None of these eight animals died as a result of the injections, even when two had multiple injections. This, taken in conjunction with a higher percentage excretion of the sucrose-injected animals, makes it evident that our evaluation of sucrose as the best diuretic by the one-hour test is also borne out by the six-hour experiments.

It can thus be definitely stated that a rabbit receiving 100 c.c. per kilogram per hour of 8 per cent sucrose in one-half strength Ringer's solution for six hours will excrete almost 90 per cent of the volume injected without serious danger to life. This represents more than half of his body weight in fluid excreted in six hours. We realize that the solutions used in these experiments

are still merely rough approximations of the ideal diuretic solution and that many experiments on different animals will have to be tried before we shall know the ideal diuretic for man.

SUMMARY

Diuresis may be obtained in rabbits by the intravenous injection of 20 per cent solutions of sucrose. One hundred cubic centimeters of this solution for each kilogram of body weight injected in one hour produced an excretion of urine approximately 130 per cent of the volume injected. Marked hydropic degeneration of the convoluted tubules occurred, and in the next twenty-four to forty-eight hours there was a marked lowering of the phenolsulfonphthalein output and temporary anuria occasionally developed. That these changes produced no permanent damage was demonstrated by repeated injections five times each week for as many as sixty-eight injections. Diuresis was unimpaired, and even though a section of the kidney removed a few days after many injections appeared irreparably damaged, within thirty-nine days the kidney on removal appeared normal grossly and microscopically and could not be distinguished from a normal kidney.

Sodium sulfate, urea, dextrose, and sorbitol injected in a similar manner in solutions with tonicity equivalent to 20 per cent sucrose produced less diuresis in every case. The histologic changes produced in the kidneys were similar in nature but definitely less than those produced by sucrose. With the exception of sorbitol all were definitely more toxic and could not be injected repeatedly as often as sucrose.

An intense diuresis was maintained for six-hour periods by the continuous intravenous injection of solutions. With sucrose solutions urine equivalent to one-half of the weight of the animal was excreted in the six-hour period. To avoid lethal effects and to maintain the excessive flow of urine it was found necessary to maintain a balance of the solution injected and the urine excreted with reference to water, sucrose, and salt. This was accomplished with a solution of 8 per cent sucrose in one-half strength Ringer's solution.

Solutions of urea, sodium sulfate, and dextrose, of tonicity equivalent to 8 per cent sucrose, in one-half strength Ringer's solution were lethal or definitely endangered the life of the animals. These solutions, as well as an equivalent sorbitol solution, produced less diuresis and permitted a greater retention of water and sodium chloride than did sucrose. Greater concentration of sorbitol produced diuresis approaching that of 8 per cent sucrose, but was fatal to three of five animals used.

REFERENCE

1. Helmholz, H. F., and Field, Ruth S.: The Kidney: A Filter for Bacteria. II. The Effect of Diuresis on the Excretion of Bacteria by the Kidney, Am. J. Dis. Child. 29: 506, 1925.

INTERRELATION BETWEEN THE VITAMIN B COMPLEX AND THE ANTERIOR LOBE OF THE PITUITARY GLAND*

DON C. SUTTON, M.S., M.D., AND JOHN ASHWORTH, M.D., CHICAGO, ILL.

THE work of Spies and others has demonstrated the specificity of nicotinic acid in the treatment of pellagra, but despite this there are some patients who fail to respond to large doses of nicotinic acid even when it is combined with liver extract and an adequate diet.

As a result of the treatment of two patients[1] reported as cachexia due to deficiency of the anterior lobe of the pituitary gland, it was noticed that pellagrous lesions improved although they had failed to improve with specific therapy.

CASE HISTORIES

CASE 1.—L. W., a 64-year-old white laborer, entered Cook County Hospital on December 26, 1937.

His immediate complaints were diarrhea, loss of weight, and edema of the feet. He was irritable and after a few days left the hospital, returning January 27, 1938 with the same complaints. He stated that a gastro-enterostomy had been done in 1915, although no evidence of such an operation can be found by either direct gastroscopy or x-rays of the stomach.

The edema of the feet had been present at various times during the past three years, and recently he had been treated at a dispensary for a cardiac condition. The remainder of the history was irrelevant.

At entrance the physical examination revealed an emaciated, weak, but not acutely ill patient. His temperature was 98.2° F., pulse 48, respirations 9 per minute, and blood pressure 126/80. An acute glossitis was noted. Examination of the chest and abdomen was negative. A gastroscopic examination revealed a reddened mucosa with a few erosions. The proctoscopic examination showed a reddened mucosa. The spinal and blood Wassermann tests were negative. The basal metabolic rate was –34. The blood chemistry showed total nonprotein nitrogen 25; urea 15.41, uric acid 2.5, cholesterol 160, sugar 86, total proteins 6.83, albumin 4.37, globulin 2.46, and ratio 1.77. There was a complete achlorhydria after an Ewald meal.

The diarrhea persisted, with five to twelve stools daily. The feces were yellow, watery, with a low specific gravity, and contained no blood. No parasites were found.

Neither the glossitis nor the diarrhea was improved by large doses of nicotinic acid, parenteral administration of liver extract, and an adequate diet. He was also treated with thyroid extract and insulin.

He lost weight (from a normal of 180 pounds to 89 pounds) and became helpless from weakness.

On October 19, 1938, daily intramuscular injections of an extract of the anterior lobe of the pituitary gland† were begun on a suspected diagnosis of a pituitary cachexia.

---

*From the Department of Medicine, Northwestern University Medical School and the Robert Bruce Preble Research Laboratory of the Cook County Hospital, Chicago.

This work has been greatly aided through a special grant from a fund in memory of Miss M. M. Capper.

Received for publication, May 15, 1940.

†The extract of the anterior lobe of the pituitary gland, "polyansyn," of Armour and Company was used. The method of preparation of polyansyn should destroy all water-soluble vitamins.

Shortly after beginning the administration of the extract of the anterior lobe of the pituitary gland, the diarrhea improved, the glossitis disappeared, and after thirty days a gain in weight and strength was apparent. By May, 1939, six months later, he weighed 167 pounds and his basal metabolic rate was −10. The pituitary extract was discontinued in May, 1939. He continued well until October 15, 1939. After a recurrence of the diarrhea and a loss of 9 pounds, 1 c.c. of the gonadotropic hormone of the pituitary gland was given intramuscularly for thirty days. The diarrhea and weight loss were promptly relieved. The gonadotropic hormone was discontinued because of the belief that it is impossible to procure an extract free of the other hormones. He continued well until April, 1940, when the diarrhea returned and later a severe glossitis.

He was allowed to lose 13 pounds after which 1 c.c. of the polyansyn was injected daily. Within forty-eight hours the diarrhea ceased, and the tongue was no longer painful. The redness disappeared in three days.

Case 2.—Mrs. T. R., a white woman 44 years old, was first seen on August 14, 1939.

Her initial complaints were weakness, loss of weight, and diarrhea. The diarrhea had been present at intervals for the past fifteen years, and continuous with several watery stools daily for the past five years.

The essentials of examination were emaciation (weight 92 pounds), a peculiar wizened facies like that seen in hypopituitary dwarfism, and a slight glossitis.

The blood examination showed hemoglobin 14.5 Gm., red blood cells 4,800,000, white blood cells 6,050, total nonprotein nitrogen 29.5, glucose 91, cholesterol 213, and chlorides 485. The basal metabolic rate was −7. Analysis of an Ewald meal revealed free hydrochloric acid 6, total acidity 22. X-ray examination of the gastrointestinal tract was entirely negative. There was a trace of blood in the stools on one occasion. No amoebae were found, although a single cyst had been reported a year previously. Because of the possibility that this was a case of atypical Simmonds' disease, she was given 1 c.c. of polyansyn intramuscularly daily. The glossitis promptly disappeared and the diarrhea improved. By February, 1940, she had gained 13 pounds, the most she has ever weighed.

The patient has since then become less emotional, is able to take care of a four-room apartment, and resume normal social activities. Her menstrual periods are less painful and have a more normal flow. Nevertheless, during each menstrual period and the week thereafter there is a moderate to severe return of the diarrhea. Recently 2 c.c. of polyansyn daily during the week preceding the menstrual period greatly decreased these recurrent diarrheas. If the polyansyn is stopped, the symptoms return within two weeks.

In view of the prompt recovery of the glossitis following the use of an extract of the anterior lobe of the pituitary gland, it appeared rational to apply this therapy to persons with pellagra who had failed to respond to the administration of large amounts of nicotinic acid, riboflavin, liver parenterally, and adequate diets.

Case 3.—C. N., a white male laborer, aged 49 years, entered the Cook County Hospital on August 29, 1939.

His immediate complaints were epigastric pain for the past year, loss of appetite for six months, and a loss of 50 pounds during the past year.

The epigastric pain had no relation to meals, although he occasionally vomited about one hour after a meal. He had a diarrhea of four to five liquid stools daily.

The essential physical findings were emaciation, a severe glossitis (a red beefy tongue), and a sore reddened pharynx.

The results of laboratory examinations showed that the urine contained a two-plus sugar and a three-plus albumin. An Ewald meal revealed an achlorhydria. No parasites nor blood were found in the feces.

A glucose tolerance test showed fasting glucose 106; at 9 A.M., 272; 10 A.M., 200; 11 A.M., 158.

The blood examination revealed hemoglobin 10 Gm., red blood cells 3,590,000, white blood cells 4,800, total nonprotein nitrogen 23, cholesterol 135, cholesterol esters 36, and phosphatase 7.38. The Kahn reaction was negative. The basal metabolic rate was −11.

X-ray of the gastrointestinal tract revealed no abnormality. A gastroscopic examination revealed a red and swollen mucosa.

On September 13, 1939, an erythematous eruption was observed on the face and in the groin. There was also maceration of the scrotum and the thighs. At this time 600 mg. of nicotinic acid daily, dilute hydrochloric acid, and insulin for the diabetes were instituted. The skin and mouth lesions showed no improvement, and on October 17, 1939, the patient was referred to the outpatient diabetic and skin clinics.

He re-entered the hospital on December 12, 1939, with typical pellagra lesions of the tongue, face, cracked scaling lesions of the lips, and corners of the mouth, symmetrical eruptions on the elbows, hands, and feet, and the groin and perianal regions. The diarrhea had become much worse. He was given 800 mg. of nicotinic acid on entrance and then 400 mg. daily until January 30, 1940. Ninety-nine milligrams of riboflavin daily were given for one week. In the meantime a positive x-ray and gastroscopic diagnosis of carcinoma of the stomach were made and all treatment was stopped.

On February 13, 1940, he was referred to our service for a trial of polyansyn. On this date the lesions were as described on December 12, 1940, and, in addition, a severe excoriation of the scrotum was found.

The diarrhea stopped after 8 c.c. of polyansyn was injected intramuscularly in daily doses of 2 c.c. At this time the patient was completely bedridden. In fourteen days he was able to walk. The scrotal, perianal, and hand and feet lesions cleared completely. The scaling of the face disappeared, but the lesions at the angles of the mouth improved about 80 per cent and the tongue about 65 per cent. The cessation of polyansyn for two to three days caused the prompt reappearance of diarrhea, glossitis, and lip lesions and the anal-scrotal and perineal lesions a few days later. One cubic centimeter of polyansyn will stop the diarrhea in twenty-four hours.

The patient has had no evidence of diabetes for the past three months. Although he is not entirely relieved, the fully 80 per cent improvement has added greatly to his comfort.

CASE 4.—Mrs. T., aged 44 years, a white waitress, was first seen in the Northwestern University hematology clinic by Doctors Howard Alt and Richard Young on December 6, 1937.

Her complaint at that time was a diffuse swelling of the right parotid gland, redness and soreness of the mouth and tongue, and painful fissures at the angles of the mouth. She had complained of vomiting and belching of gas for fifteen years prior to her admission to the clinic. In 1927 she was examined at the Mayo Clinic.

No organic disease was found and it was then suggested that she had a vitamin deficiency.

Until November, 1939, she was treated for a secondary anemia and a vitamin deficiency. Iron, yeast, meat, oranges, and grapefruit were added to a supposed liberal diet, but her low income prevented her strict adherence to it. From November 21, 1939, 5 mg. of riboflavin and 300 mg. of nicotinic acid daily were also given. On January 6, 1940, the nicotinic acid was increased to 900 mg. daily for two weeks. From March 6 to March 16, 1940, 20 Gm. daily of ventriculin were given. On March 16, 1940, she was referred to us. At that time her tongue was red and painful. She had painful cracks at the corners of her mouth, with scaling of the skin at the lip borders. These lesions had not completely healed in three years.

She was given 2 c.c. of polyansyn daily. At the end of five days the mouth was entirely healed and the tongue was less red and painful. She continued well with 10 c.c. of polyansyn weekly until one week before her menses, when the angles of her mouth once more became sore and cracked, and her tongue became red and painful. The injections were continued and after menstruation ceased, the lesions promptly disappeared.

With the next menses the angles of the mouth became painful but did not crack. The condition disappeared before the menses were completed, and the menses became less painful during treatment. When last seen, May 10, 1940, she was entirely well.

CASE 5.—C. F., a Chinese male, 54 years old, entered the Cook County Hospital with a moderate cardiac decompensation, due to an aortic stenosis of rheumatic origin.

He showed no other significant findings, except a symmetrical dry scaling eruption on the backs of the hands, the buttocks, and the thighs. These lesions were diagnosed as pellagra. He was treated for his cardiac condition but kept on a vitamin-poor diet for ten days. At

J. LAB. & CLIN. MED.
VOL. 25, NO. 11, AUG. 1940

PLATE I. Case 4.—Before treatment with polyansyn. Note cheilosis lesion in angle of the mouth.

PLATE II. Case 4.—After treatment with polyansyn.

UNIV. OF MICH.

the suggestion of Dr. Dunbar he was given 1,000 c.c. of glucose intravenously on March 20. The next day all the lesions were acutely inflamed and painful, and the backs of his hands were cracked and exuded serum.

He was continued on the low vitamin diet and given 2 c.c. of polyansyn daily. Improvement was prompt, and on May 9 all evidence of scaling was gone.

DISCUSSION

With the exception of Case 5, all patients had been well treated as pellagrins and yet showed little or no improvement for long periods of time. The use of an extract of the anterior lobe of the pituitary gland, as already stated, was suggested by the prompt improvement of the glossitis and diarrhea in Cases 1 and 2. These cases were considered as probably pituitary cachexias. The other cases had no evidence of pituitary hypofunction and yet improved with the administration of polyansyn. The lesions improved by the use of polyansyn are the same as those accepted as being due to deficiency of nicotinic acid and riboflavin. There is some evidence that starvation or vitamin B deficiency may lead to damage of the anterior lobe of the pituitary gland.

Marburg and Wenchebach observed necrotic areas in the anterior lobe of the pituitary glands of patients dying from beriberi.

Burke and McIntyre[3] found that rats on a thiamin-deficient diet did not lose weight as rapidly when given a growth-promoting extract of the pituitary as did those who did not receive the extract; they concluded that the growth-promoting hormone modifies the utilization of thiamin.

Brenneman[4] found that when day-old chicks were fed a standard diet only on alternate days growth was retarded to about 50 per cent of that of normal controls fed the same diet every day. The testes were lighter in the starved chicks. Male hormone limited the growth of the testes of the control chicks but not of the starved chicks. This observation is interpreted as evidence of decreased production of the gonadotropic hormone. This is further suggested by the fact that injection of an extract of the anterior lobe of the pituitary gland produces greater growth of the testes and combs in the starved chicks than in normal controls.

Sommer[5] found that women who had passed through puberty during the starvation caused by the blockade of Germany during 1914 to 1918 showed a high incidence of abortions and stillbirths. Delayed menstruation, amenorrhea, infantilism, and sterility were common. Corpus luteum hormones failed to cause improvement. Prolan benefited many cases. Greatly diminished lactation was common and was greatly improved by the use of lactogenic hormone. From these observations he concluded that the lactogenic and gonadotropic hormones of the pituitary gland were diminished by starvation.

Hundhausen[6] found that deprivation of vitamin B_1 caused a decrease in the thyrotropic and gonadotropic hormones in rats.

From this experimental work it appears that vitamin deficiency or partial starvation may cause a decrease of the hormones of the anterior lobe of the pituitary. This may serve to explain the results in all cases except Case 5. In this case, recovery occurred with the administration of polyansyn, although a low vitamin diet was continued. An attempt to answer some of these questions is now being done through a series of laboratory observations.

CONCLUSION

Evidence is presented indicating that an extract of the anterior lobe of the pituitary gland will cause recovery of pellagra lesions when they have failed to respond to nicotinic acid, riboflavin, parenteral liver, and adequate diet.

REFERENCES

1. Sutton, Don C., and Ashworth, John: Cachexia Responding to Extract of the Anterior Lobe of the Pituitary Gland, J. LAB. & CLIN. MED. **25**: 848, 1940.
2. Marburg, O.: Über Veränderungen der Hypophyse bei Beriberi (Ein Beitrag zur Kenntnis der basophilen Hypophysen-Zellen), Wien. Arch. f. inn. Med. **29**: 1, 1936. Quoted by Van Dyke: Physiology and Pharmacology of the Pituitary Gland, Chicago, 1939, Univ. of Chicago Press.
3. Burke, J. C., and McIntyre, A. R.: Growth Promoting Pituitary Extract in Thiamine Deficiency, Proc. of the 32nd Annual Meeting of the Am. Soc. of Biol. Chem. March 30 to April 2, 1938.
4. Brenneman, W. R.: Limitation of Food Consumption as a Factor Influencing Endocrine Reactions in the Chick, Waterman Institute and Zoology Dept., Univ. of Indiana. Personal communication.
5. Sommer, K.: Hormonale Unterfunktion als Hungerfolge, Arch. f. Gynäk. **166**: 204, 1938.
6. Hundhausen, Gerhart: Über die Beziehungen zwischen Avitaminose-B_1 und gonadotropen Hormon des Hypophysenvorderlappens, Arch. f. exper. Path. u. Pharmakol. **192**: 670, 1939.

LABORATORY METHODS

MODIFICATION OF THE GALACTOSE TOLERANCE TEST BASED ON THE DIFFERENTIAL FERMENTATION OF GLUCOSE OCCURRING WITH GALACTOSE IN URINE*

S. S. LICHTMAN, M.D., NEW YORK, N. Y.

THE galactose tolerance test continues to be used as a standard diagnostic procedure in cases of jaundice since its introduction by Bauer.[1] Few of the many tests for the study of liver function that have since been introduced enjoy such wide application. The diagnostic value and the simplicity of the procedure account for its widespread routine use.

It has been repeatedly affirmed that this test is of limited value if applied as a quantitative test of liver function (Lichtman, 1931[2]; Shay and Schloss, 1931, 1932[3]). It is, however, valuable in the differential diagnosis of jaundice. The procedure has been modified with the aim of increasing its diagnostic value. Thus galactose has also been estimated in the blood after either oral or intravenous administration of galactose (Jankelson, 1937[4]). Recently, Shay and Fieman (1937)[5] have also recommended a modification of the routine technique: differential fermentation of the urine for galactose and glucose. This addition seemed especially advantageous to them in the performance of the galactose tolerance test in patients with disturbed endocrine states, i.e., thyroid, adrenal, pituitary, and diabetes, where false positives might occur due to associated disturbances in glucose tolerance.

I have for some time contemplated the routine fermentation of the individual fractions after the oral administration of galactose. Bauer[6] originally attempted to identify the reducing substance in the urine as galactose after feeding this sugar. He applied the standard mucic acid test for this sugar to this purpose. This method, however, permitted a maximum recovery of only 70 per cent of galactose added to urine. Thus, part of the remaining 30 per cent of reducing substance in the urine after galactose feeding might possibly occur in the form of a sugar other than galactose. He discarded fermentation of the urine by yeast for the purpose of identification because of equivocal results he obtained in yeast fermentation of galactose.

The nature of the reducing substance which appears in the urine following the oral administration of galactose under pathologic conditions merits further study. Studies on the metabolism of galactose have indicated a conversion to glycogen both in animals and man (Roe, Gilman, and Cowgill, 1934;[7] Shay, Schloss, and Bell, 1931[8]). A rise in blood glucose may follow the ad-

*From the Laboratories and the Medical Division, Service of Dr. George Baehr, the Mount Sinai Hospital, New York.
Received for publication September 26, 1939.

ministration of galactose (Blanco, 1928;[9] Block and Weiss, 1930;[10] Harding and Grant, 1933[11]). Furthermore, in diabetes, blood glucose was found by Roe and Schwartzman (1932)[12] to be increased after galactose ingestion. Roe and Cowgill (1935)[13] noted a fall in blood galactose concomitantly with a gradual steady increase in blood glucose in rabbits following galactose administration. Thus, there is ground for the suspicion that under certain pathologic conditions the rate of the conversion of galactose to glucose and the tolerance for glucose may be impaired, so that some glucose may appear in the urine besides galactose.

It would be inadvisable to introduce any modification of the original technique which would change it from a convenient routine laboratory procedure into a complicated one. The differential fermentation of sugar, fortunately, is a simple procedure and requires no special apparatus or technique. The modification of the technique here proposed guards against possible sources of error. The problem involves a knowledge of the behavior of the yeast cell in relation to mixtures of fermentable and nonfermentable sugars. Under ordinary conditions baker's yeast does not ferment galactose.* If galactose disappears from a solution after contact with a yeast suspension, phenomena other than fermentation, such as adsorption and diffusion, must be suspected. Sobotka, Holzman, and Reiner (1936)[14] have noted different rates of diffusion of two components of a sugar mixture. According to the speed of diffusion into the yeast cell, the sugars may be arranged in the following order: xylose > glucose > galactose > lactose. Sugars may leave solutions by diffusion into yeast cells without actual fermentation, as this occurs even when alcoholic fermentation is suppressed (by toxic agents).

While small amounts of a fermentable sugar, assumed to be glucose, may appear in the urine after galactose administration, according to the results of the present study, an error may arise if the diffusion of galactose into yeast cells in the fermentation of galactose-glucose mixtures is not first taken into account. Another source of error lies in the calculation when the reduction factor for galactose* is applied to a fraction which is actually glucose.

PROCEDURE

The standard technique, as described by Bauer, was followed. The fractions were tested qualitatively for copper reduction and were then estimated quantitatively with Benedict's or Rudisch's solution.†

The latter solution boils with a minimum of bumping. The standard correction factor of 0.77 was applied for galactose. Fresh baker's yeast was employed. The yeast was washed if the supernatant contained a reducing substance. It is necessary to limit the amount of yeast to 5 per cent by volume of the solution to be fermented. Approximately equal amounts of yeast were used in all the fermentations which were carried on at temperatures between 40° and 45° C. for a period of sixty minutes. Controls consisted of a 1

*Only after prolonged contact may baker's yeast be adapted to the fermentation of galactose.

†An alkaline copper solution recommended by Rudisch for the quantitative estimation of glucose in urine (International Contributions to Medical Literature, Festschrift in honor of Dr. A. Jacobi, 1900).

per cent solution of glucose in distilled water and a solution of between 1 and 2 per cent of galactose in normal urine, free of glucose. Sugar estimations were made on the supernatant liquid after centrifugation of the yeast suspension. The glucose control served to prove the activity of the yeast suspension.

CALCULATIONS

Galactose has a lower reducing power than glucose. 1.3 Gm. of C.P. galactose ($[\alpha]D = +80.5°$) are equivalent to 1.0 Gm. of glucose in the reduction of equivalent amounts of Benedict's or Rudisch's quantitative sugar reagents.

The sugar content is determined before and after fermentation. The non-fermentable fraction is considered to consist of galactose. A correction is made for the galactose which may have disappeared from the mixture as determined by the galactose control. The fermented fraction represents the glucose concentration. Since the sugar content before fermentation was calculated on the basis of the assumption that all the sugar in the test urine was galactose, the figure obtained by subtracting the sugar concentration after fermentation from that before fermentation must be divided by 1.3 to convert this assumed galactose into a glucose value. For example:

	BEFORE FERMENTATION	AFTER FERMENTATION	GRAMS GLUCOSE
Control galactose solution	1.5%	1.4% (0.1%)*	—
100 c.c. test urine	1.9%	1.6% (1.7%) (Corrected)	0.30 (0.20)† (Corrected)

*Correction for galactose diffusion into yeast suspension.

†Fermentable sugar divided by 1.3 to give glucose value $\frac{0.20}{1.3} = 0.15$ Gm. glucose. (One gram of glucose is equivalent to 1.3 Gm. of galactose in the reduction of Benedict's and Rudisch's solution.)

RESULTS

In 80 tests made on patients with suspected liver disease with normal and disturbed galactose tolerance, fermentation over a period of twenty-four hours disclosed a disappearance of reducing substance varying between 0.1 and 0.7 per cent, in the majority of instances, 0.3 per cent. Under these circumstances the "fermentable" fraction was found to constitute as much as 30 to 40 per cent of the total reducing substance in the urine. However, in this series corrections were not made for diffusion of galactose into the yaest suspension over a period of twenty-four hours. In clinical laboratories this correction has not customarily been made when glucose-galactose mixtures have been fermented, leading in diabetic urine to erroneous conclusions concerning the amount of "fermented" glucose present.

In 12 instances the disappearance of reducing substances from a galactose solution added to normal urine was compared at the end of one hour and at the end of twenty-four hours of fermentation. At the end of one hour there was no change in 9 of the 12 tests. The amount that disappeared in the 3 remaining tests were 0.10, 0.10, 0.13 per cent, respectively. At the end of twenty-four hours these same tests showed a decrease in reducing substance varying from 0.10 to 0.38 per cent.

TABLE I

MODIFIED GALACTOSE TOLERANCE TEST IN 10 PERSONS WITH SUSPECTED LIVER DAMAGE

CASE NO.	GALACTOSE URINE CONTROL PER CENT LOSS	1ST HOUR PER CENT			2ND HOUR PER CENT			3RD HOUR PER CENT			4TH HOUR PER CENT			TOTAL GRAMS SUGAR GALACTOSE‡			DIAGNOSIS
		B.F.*	A.F.†	NET LOSS	B.F.	A.F.	NET LOSS	B.F	A.F.	NET LOSS	B.F.	A.F.	NET LOSS	BAUER	A.F.	GLUCOSE	
1	0	1.37	1.37	0	5.5	4.7	0.8	2.54	2.3	0.24	0.8	0.8	0	6.67	5.93	0.64	Toxic hepatitis
2	0	1.30	1.22	0.08	0.97	0.87	0.10	0	–	–	0	–	–	1.36	1.25	0.09	Graves' disease
3	0	1.76	1.62	0.14	–	–	–	–	–	–	1.17	1.04	0.13	4.57	4.20	0.28	Portal cirrhosis
4A	0.03	3.5	3.2	0.27	2.90	2.64	0.23	1.5	1.38	0.09	0	–	–	5.46	5.03	0.32	Toxic cirrhosis
B	0	4.8	4.25	0.55	1.98	1.89	0.09	0.92	0.92	0	0	–	–	6.75	6.32	0.34	
5	0	1.95	1.3	0.65	3.2	2.26	0.94	2.6	1.5	1.1	1.6	1.05	0.55	5.69	3.65	1.58	Choledocholithiasis; diabetes mellitus
6	0	1.3	1.3	0	0.81	0.81	0	0.74	0.74	0	0	–	–	2.69	2.69	0	Toxic hepatitis (recovery)
7	0.12	1.58	1.38	0.08	1.44	1.32	0	0	–	–	0	–	–	4.8	4.73	0.05	Infectious jaundice
	0	0.37	0.37	0	0.75	0.60	0.15	0	–	–	0	–	–	2.23	2.05	0.14	
8	0	1.46	1.46	0	1.05	1.05	0	0	–	–	0	–	–	1.41	1.41	0	Graves' disease
9	0	1.6	1.6	0	0	–	–	0	–	–	0	–	–	4.56	4.56	0	Carcinoma of gallbladder; jaundice
10	0.03	1.86	1.72	0.11	0	–	–	0	–	–	0	–	–	2.80	2.64	0.12	Pigment cirrhosis

*B.F. = Before fermentation.

†A.F. = After fermentation.

‡$\frac{\text{B.F.-A.F.}}{1.3}$

In 12 tests observations were made on the disappearance of reducing substance from control galactose solutions added to urine at the end of fermentation for one hour only. In this series there was only a single instance of disappearance of reducing substance, in which case 0.12 per cent disappeared.

The percentage decrease of reducing substance at the end of one hour in 24 tests occurred as follows: In 21 instances there was no change; in the 3 remaining instances decreases of 0.10, 0.10, and 0.12 per cent, respectively, were found. In 12 tests observed at the end of twenty-four hours, 6 showed decreases of 0.10 to 0.20 per cent; 2, decreases of 0.20 to 0.30 per cent; and 4, decreases of 0.30 to 0.38 per cent.

In Table I are listed the results in 10 cases of jaundice or disease associated with liver damage. In 4 cases the galactose tolerance was normal, i.e., total excretion was less than 3.0 Gm. In 3 instances the reducing substance found in the urine was found to occur entirely in the form of galactose. Two were in normal tests, the third in a pathologic galactosuria. In 2 tests less than 0.1 Gm. of fermentable substance was present. In the remainder, the amount of glucose present varied between 0.12 Gm. to 0.65 Gm. In a single instance (Case 5) 1.58 Gm. of glucose was present. In this case there was an associated diabetes. The pathologic galactosurias in the series varied between 3.65 Gm. and 6.32 Gm. Excluding the glycosuria associated with diabetes, glycosurias of 0.28, 0.32, 0.34, and 0.64 Gm. were encountered in patients with galactosurias of 4.20, 5.03, 6.32, and 5.93 Gm., respectively. Glycosuria was noted in individual fractions only, and in Case 4 (Test A) in every fraction of urine containing galactose.

COMMENT

Experimental studies in galactose tolerance in man and animals have indicated that a reducing substance other than galactose may occur in the urine following the oral and intravenous administration of galactose.[7-13] Under pathologic conditions involving the liver this tendency might be expected to be even more marked. It is difficult to conclude whether the glycosurias associated with galactosuria are primarily the result of the conversion of galactose to glucose or whether the glucose voided in the urine is the result of a displacement of this sugar in the liver following galactose ingestion.

While the amounts of glucose voided in the urine have not been large enough in this small series to alter appreciably the results of the galactose tolerance test, the modified procedure may prove to be of especial diagnostic significance in borderline cases, with values of 3.0 to 4.0 Gm. of galactose.

The galactose control solution is necessary in the accurate estimation by fermentation of sugars in galactose-glucose mixtures. Failure to account for amounts of galactose which diffuse into yeast cells leads to erroneous conclusions favoring the amounts of glucose present in such mixtures. This caution applies especially to the performance of the galactose tolerance test in subjects with diabetes mellitus. The modification introduced here of limiting the period of fermentation to sixty minutes at elevated temperature reduces the rate of diffusion of galactose into yeast cells practically to nil, and at the same time permits the complete fermentation of as high as 2.5 per cent of glucose.

SUMMARY

A modification of the galactose tolerance test is introduced without interfering with its simple routine clinical use in persons with suspected liver disease.

The modification depends upon the selective fermentation of glucose in galactose-glucose mixtures; under the conditions of the test, galactose being nonfermentable. The possible disappearance of galactose by diffusion into yeast cells is taken into account. The fermented fraction then represents glucose.

Glucose may appear in the urine, associated with normal and pathologic alimentary galactosuria. There is a tendency for increasing amounts of glycosuria to occur with increasing alimentary galactosuria.

The modified technique improves the accuracy of estimation of mixtures of galactose and glucose present in urine in the routine application of the galactose tolerance test in persons with suspected liver damage and with diabetes mellitus.

REFERENCES

1. Bauer, R.: Ueber alimentaere Galaktosurie bei Ikterus, Deutsche. med. Wchnschr. **34**: 1505, 1908.
2. Lichtman, S. S.: Cinchophen Oxidation Test of the Function of the Hepatic Cells, Arch. Int. Med. **48**: 98, 1931.
3. Shay, H., and Schloss, E. M.: The Galactose Tolerance Test in the Differential Diagnosis of Jaundice, Arch. Int. Med. **47**: 650, 1931; Painless Jaundice, Its Differential Diagnosis by the Galactose Tolerance Test, J. A. M. A. **98**: 1433, 1932.
4. Jankelson, I. R.: Intravenous Galactose Liver Function Test, Am. J. Digest. Dis. & Nutrition **3**: 889, 1937.
5. Shay, H., and Fieman, P.: The Galactose Tolerance Test in Jaundice: A Consideration of the Evidence Permitting the Measurement of Galactose Utilization by Urinary Excretion; Some Sources for Error in Its Interpretation; and An Addition in Routine Technic, Ann. Int. Med. **10**: 1297, 1937.
6. Bauer, R.: Eine expedition Methode aum Nachweis von Galaktose und Milchzucker im Harn, Ztschr. f. physiol. Chem. **51**: 158, 1907.
7. Roe, J. H., Gilman, A., and Cowgill, G. R.: A Study of the Oxidation That Occurs in the Dog and in Man After Ingestion of Galactose, Am. J. Physiol. **110**: 531, 1935.
8. Shay, H., Schloss, E. M., and Bell, M. A.: The Metabolism of Galactose. I. Considerations Underlying the Use of Galactose in Tests of the Function of the Liver, Arch. Int. Med. **47**: 391, 1931.
9. Blanco, J. G.: Sugar Metabolism; Lactose, Galactose and Xylose, J. Biol. Chem. **79**: 667, 1928.
10. Block, J., and Weiss, M.: Reflex-oder Resorptionshyperglykämie? Ztschr. f. d. ges. exper. Med. **69**: 453, 1930.
11. Harding, V. J., and Grant, G. A.: Metabolism of Galactose. I. Cutaneous Blood Sugars After Galactose Ingestion, J. Biol. Chem. **99**: 629, 1933.
12. Roe, J. H., and Schwartzman, A. S.: Galactose Tolerance of Normal and Diabetic Subjects and the Effect of Insulin Upon Galactose Metabolism, J. Biol. Chem. **96**: 717, 1932.
13. Roe, J. H., and Cowgill, G. R.: The Metabolic Fate of Galactose in Adult Dogs and Rabbits, Am. J. Physiol. **111**: 530, 1935.
14. Sobotka, H., Holzman, M., and Reiner, M.: Selective Fermentation. III. Fermentation of Hexose-Pentose Mixtures, Biochem. J. **30**: 933, 1936.

A METHOD FOR THE QUANTITATIVE ESTIMATION OF CHEMICAL IRRITATION*

J. H. WEATHERBY, PH.D., RICHMOND, VA.

IN MANY biological studies it is frequently desirable to measure quantitatively the irritating properties of chemicals. Heretofore, one has had to depend largely on a personal sensation of pain and on intensities of hyperemia and inflammation produced by irritants, with only one's memory of previous observations upon which to base a comparison. Such is the method described by Munch.[1] In the absence of a more accurate method, information obtained in such a manner may be of considerable value. However, memory is at best an unreliable faculty; and from a scientific point of view, data in which memory of previous experiences plays a significant part are of limited value because of the difficulty of independent repetition and verification. A few investigators have made use of various physical means for measuring the intensity of inflammation, relying on hyperemia or edema to provide an index of the severity of the reaction. While the degree of redness of an inflamed area can be measured with satisfactory accuracy, as described by Oettel,[2] it is not established that redness is a constant and reliable index of the degree of inflammation. Moreover, such measurements usually require elaborate and expensive apparatus. Further, it is only under specialized conditions that redness bears a quantitative relationship to the inflammatory process with which it is associated. The use of edema as a criterion is likewise subject to many objections, among which is the very great variation in results obtained when the same chemical is applied to the same type of tissue. Thus, Haag[3] found very great variations in the amounts of edema produced by irritants instilled into the conjunctival sacs of rabbits' eyes. Also, the same solution of an irritant frequently produced no visible edema in the eyes of one rabbit, but marked edema in those of another.

Ebbecke,[4] Hirschfelder,[5] and Menkin[6] observed that trypan blue, when injected intravenously, becomes fixed at all sites of inflammation. Regardless of the exact mechanism of this fixation, if the intensity of the blue color resulting from the accumulation of dye at the site of inflammation is proportional to the intensity of inflammation, a potential method is at hand by which the relative strengths of chemical irritants may be measured quantitatively. Tainter, Throndson, and Lehman[7] applied this principle in a very limited number of observations, but apparently did not extend their observations to include more than a single chemical, sodium bisulfite.

*From the Department of Pharmacology, Medical College of Virginia, Richmond.
Received for publication, October 25, 1939.

In these experiments a variety of chemicals was used, both as solutions of a single substance and in mixtures. Up to the present time well over fifteen hundred rabbits have been used for these observations. The results from a few representative chemicals are reported here.

EXPERIMENTAL METHODS

Adult white rabbits of both sexes were used as test animals throughout these experiments. Gray or brown rabbits were found unsatisfactory because the skin pigments partially obscured the dye. Solutions of irritants, the irritating properties of which were to be studied, were injected subcutaneously into the dorsal surfaces of the ears, usually at three sites on each ear. Intracutaneous injections into the skin of the abdomen were tried, but were found less satisfactory for two reasons: first, the skin of the abdomen seemed to be less sensitive to the action of irritants; and second, slight trauma to the skin caused by removing the fur either by shaving or by using barium sulfide as a depilatory, was frequently sufficient to cause the accumulation of so much dye in the skin as to interfere with the evaluation of the intensity of spots at the sites of injection of the solutions being tested. The intravenous injection of the dye was made into one of the superficial veins of the abdomen in most instances, but occasionally into one of the marginal veins of the ear. It was considered advisable to avoid injections into ear veins as far as possible, lest manipulation and trauma interfere in some way with the uniform distribution of dye in the two ears. The various irritant solutions were injected in $\frac{1}{20}$ c.c. quantities. The amount of trypan blue used was 1 c.c. per kilogram of 1 per cent solution in 0.85 per cent sodium chloride. This quantity of dye was much less than that used by Menken, but proved to be adequate. Moreover, larger quantities of dye frequently resulted in such intense coloration at the sites of inflammation that comparison with a standard color scale became difficult or even impossible. The standard color scale was prepared by placing drops of solutions of various concentrations of the dye on a white background, for which blotting paper was found satisfactory. In the scale used the concentration of dye varied from a maximum of 0.05 per cent to a minimum of 0.0029 per cent. There were eight different concentrations in the scale, and each was two-thirds as high as the one above it. Thus, No. 8 was 0.05 per cent, No. 7 was 0.033 per cent, No. 6 was 0.022 per cent, and so on.

It was found that solutions of different concentrations of an irritant produced blue spots which, within a limited range, were proportional in intensity to the concentrations of the solutions. However, the maximum range of color between a slight, though definite, blueness, and an intensity beyond which no further increase could be noted, corresponded to only a relatively limited range of concentration of the irritant. Also, it was found that any given solution of an irritant produced spots which varied in intensity over a range of two or three points on the standard scale. Therefore, in order to have the method yield quantitative results it was necessary to assign to each degree of intensity on the color scale an arbitrary numerical value. It was found experimentally that if these arbitrary numerical values were propor-

tional to the concentration of dye in the standard scale, the degree of irritation, as indicated by this numerical value, was approximately proportional to the concentration of the irritant solution. Thus, doubling the concentration of the irritant solution gave values which were approximately doubled. Frequently traces of dye were observed at the sites of injection which were less than the lowest intensity on the color scale. These were designated as "traces," and assigned a value in keeping with the remainder of the scale. The arbitrary values selected are as follows: Trace, 0.66; No. 1, 1.0; No. 2, 1.50; No. 3, 2.25; No. 4, 3.37; No. 5, 5.06, etc. Concentrations of irritant solutions were selected which gave values rarely as high as No. 4 (3.37). With such concentrations the values ordinarily varied between 0.66 and 2.25, with an average for several animals of 0.9 to 1.5.

Observations extending over a period of about eighteen months indicate that certain climatic conditions, particularly temperature, greatly influence the intensity of the blue spot. This is to be expected when it is considered that circulation through vessels in the rabbit's ear apparently changes greatly with changes in temperature. This fluctuation between peripheral vasoconstriction and vasodilatation was observed to produce results varying by as much as 100 per cent. Presumably air-conditioned animal rooms and laboratories would greatly reduce such fluctuations, but in the absence of such uniform conditions of temperature and humidity it is necessary to compensate in another manner. The procedure found to give satisfactory results is to inject a standard irritant solution into one ear of the animal and the unknown solution into the other. Then, by dividing the average value for the unknown solution by that for the standard solution a quotient is obtained which represents the ratio, Unknown/Standard. The standard irritant solution routinely used contains 0.1 N acetic acid, 0.1 N sodium acetate, and 0.1 per cent benzoic acid. This mixture has a pH of 4.68; it is stable and easily reproducible. Immediately before use it is diluted to four volumes, so as to contain 0.025 N acid and salt, and 0.025 per cent benzoic acid. Triply distilled water was used in the preparation of all solutions.

It was found that various nonirritant substances, such as 0.85 per cent sodium chloride solution and liquid petrolatum, caused the accumulation of traces of dye at the sites of injection if the dye was administered immediately after the injections were made. However, if a period of about forty minutes was allowed to elapse between the injection of irritant and the injection of dye, there was no significant accumulation of dye following the injection of 0.85 per cent sodium chloride solution. It was, therefore, uniformly the practice to wait forty minutes after injections of irritants before giving the dye. Liquid petrolatum caused the accumulation of dye even if the usual period of forty minutes was extended to two or three hours; for this reason, it was not used as a solvent in any of the experiments reported here. Occasionally, though rarely, sufficient trauma was produced by the injection to give a faintly positive reaction, even though a nonirritant solution was used. This was minimized by the use of hypodermic needles of not larger than 27 gauge. Occasionally small blood vessels were damaged by the injection, so as to result in a small hemorrhagic spot which contained little or no dye. Such spots

were omitted in the final observations. Injections of distilled water uniformly produced a definite accumulation of dye, so all solutions were made isotonic. Following the injection of irritant solutions, the accumulation of dye was usually evident within ten or fifteen minutes, and reached maximum intensity within forty-five minutes, at which time final comparison with the standard color scale was made. The persistence of the blue spots was more or less proportional to the degree of inflammation. Spots from barely irritating concentrations were usually gone within twenty-four hours, but if the concentration of the irritant solution was sufficient to produce spots corresponding to 7 or 8 on the standard scale, they usually persisted for several days, or even weeks. No attempt was made to measure persistence of spots with greater accuracy because of the interference from dye which always accumulated in subcutaneous tissues after eighteen to twenty-four hours.

For approximate results 3 or 4 animals were usually sufficient, and the standard irritant solution was omitted. For more accurate results 16 animals were found to suffice when the standard irritant solution was used in one ear of each animal. Analyses by statistical methods of results so obtained indicate that differences of the order of 15 to 20 per cent can be detected with a degree of certainty of 99 per cent, which compares favorably with many other accepted methods of bio-assay.

Due to the persistence of the dye in tissues of animals they cannot be used a second time within less than about six weeks after the first experience.

TABLE I

1 SUBSTANCE	2 MOLAR CONC.	3 UNKNOWN IRRITATION VALUE	4 STANDARD IRRITATION VALUE	5 UNKNOWN / STANDARD	6 CORRECTED MOLAR CONC.	7 CORRECTED UNKNOWN STANDARD
HCl	0.001	1.07 ± 0.056	1.39 ± 0.060	0.77 ± 0.050	0.0013	0.82 ± 0.067
CH_3COOH	0.003	0.91 ± 0.050	1.49 ± 0.060	0.61 ± 0.040	0.0049	0.81 ± 0.029
NH_4OH	0.02	0.91 ± 0.042	1.30 ± 0.042	0.70 ± 0.039	0.0286	0.81 ± 0.047
HCHO	0.04	0.79 ± 0.036	1.10 ± 0.043	0.72 ± 0.045	0.0556	0.89 ± 0.044
$NaHSO_3$	0.015	0.95 ± 0.041	1.38 ± 0.043	0.69 ± 0.054	0.022	0.88 ± 0.046

RESULTS

Substances studied according to the method described herein include acids, bases, salts, glycols, and formaldehyde. Results are shown in Table I. In this table are included molar concentrations of irritant solutions used (2); average irritation values of solutions, as obtained from observations on 16 animals for each (3); average irritation values of the standard irritant solution obtained simultaneously (4); the ratio unknown/standard (5); predicted molar concentrations of irritant solutions which should give a ratio unknown/standard of 1 (6); and the ratio unknown/standard actually obtained from these predicted molar concentrations (7). Figures in column 6 were obtained by dividing molar concentration actually used (2) by unknown/standard (5). Therefore, corrected molar concentrations (6) represent concentrations which should be equally irritating among themselves and also with the standard solution. In column 7 are shown figures obtained from the corrected molar concentrations.

These figures uniformly approach those for the standard more closely. With formaldehyde the error is well within the limits of the method—15 to 20 per cent—but with acids and ammonia the agreement is not as close as might be desired. A third experiment, the results of which are not shown in the table, was performed with ammonia. A second corrected molar concentration was calculated just as was done for the first, except that the calculation was based on corrected unknown/standard of 0.81 instead of on unknown/standard of 0.70. The solution prepared on the basis of this calculation gave a ratio for unknown/standard of 1.00, which is in perfect agreement with the theoretical value.

Two possible explanations may be offered for the lack of better agreement between actual and predicted values for the acids and ammonia: (1) intensity of the blue color is not exactly proportional to concentration of the irritant solution; and (2) in spite of the fact that triply distilled water was used throughout these experiments in preparing solutions, sufficient buffering material may have been present to alter the hydrogen-ion concentration to a significant extent. Frequent pH measurements with a glass electrode indicated that it is difficult to prepare such dilute solutions of electrolytes and not have the pH altered slightly by traces of buffering substances which are so nearly impossible to remove. The second explanation appears even more probable in view of the fact that when the standard solution at a concentration of 0.015 N acid and salt was compared with the same standard at the usual concentration of 0.025 N, the ratio was 0.66. When a concentration of 0.020 N was compared in the same manner, the ratio was 0.81. These two ratios are to be compared with theoretical ratios of 0.60 and 0.80, assuming a direct proportion between concentration and intensity of blueness. It is obvious that a buffered solution, such as the standard, would be much more stable in the presence of traces of buffering material than dilute solutions of acids and ammonia, such as those used.

Sodium hydroxide appeared to be two or three times as irritating as ammonium hydroxide, although reliable figures were difficult to obtain. At concentrations less than 0.01 N there appeared to be little accumulation of dye; at higher concentrations the usual effect was production of a blue ring about one-half inch in diameter, having a clear area in the middle. It appeared as though there was tissue destruction at the site of injection. Due to this atypical appearance strong bases were not investigated further.

Acetates and formates of nicotine and ammonia showed practically no irritating properties in concentrations less than isotonic, and are, therefore, not included in the table. Sodium bisulfite forms a relatively strongly acidic solution, and acts similarly to other buffered solutions, such as the standard. The results obtained with sodium bisulfite confirm those reported by Tainter, Throndson, and Lehman. The glycols (propylene glycol, diethylene glycol, and glycerin) produced no significant degree of inflammation at concentrations less than 2 or 3 moles per liter. Since isotonic concentrations of nonelectrolytes are usually considered to be about 0.29 molar, one is justified in con-

cluding that irritation from such concentrations of glycols as the above was the result of hypertonicity rather than of inherent chemical properties of these substances.

Numerous tests on the relative irritating properties of solutions of cigarette smoke prepared from cigarettes containing glycerin as the hygroscopic agent and those containing diethylene glycol failed to show a significant difference between the two types. Smoke solutions were prepared according to the gravity collection method described by Bradford, Harlan, and Hanmer.[8] All experiments were conducted in such a manner that the operator was not aware of the identity of the solutions being tested.

REFERENCES

1. Munch, J. C.: Manual of Biological Assaying, Philadelphia, 1937, J. B. Lippincott Co., p. 115.
2. Oettel, H.: Einwirkung organischer Flüssigkeiten auf die Haut, Arch. f. exper. Path. u. Pharmakol. **183**: 641, 1936.
3. Haag, H. B.: Unpublished data.
4. Ebbecke, U.: Capillarerweiterung, Urticaria, und Schock, Klin. Wchnschr. **2**: 1725, 1923.
5. Hirschfelder, A. D.: Studies Upon the Vascular and Capillary Phenomena and Supposed Axon Reflexes Concerned in the Development of Edema in Mustard Oil Conjunctivitis, Am. J. Physiol. **70**: 507, 1924.
6. Menkin, Valy: Studies on Inflammation, J. Exper. Med. **50**: 171, 1929.
7. Tainter, M. L., Throndson, A. H., and Lehman, A. J.: Local Irritation From Sodium Bisulphite as Preservative in Epinephrine Solutions, Proc. Soc. Exper. Biol. & Med. **36**: 584, 1937.
8. Bradford, J. A., Harlan, W. R., and Hanmer, H. R.: Nature of Cigaret Smoke: Technic of Experimental Smoking, Indust. & Engin. Chem. **28**: 836, 1936.

ESTIMATION OF THIOCYANATES IN THE BLOOD WITH THE USE OF PERMANENT STANDARDS*

ABE RAVIN, M.D., DENVER, COLO.

RECENT reports indicate that thiocyanate therapy may be a useful remedy for certain types of hypertension, provided the treatment is properly controlled by means of blood thiocyanate determinations.[1-3] For clinical uses the method for blood thiocyanate determination to be described presents one or more of the following advantages over previously described methods: (1) No expensive equipment is necessary. (2) Repeated preparation of standard solutions is eliminated. (3) A determination can be made in a few minutes. (4) A determination can be made on 1 c.c. or even 0.25 c.c. of serum.

MATERIALS

1. Twenty per cent trichloracetic acid solution.

2. Ferric nitrate reagent: Dissolve 5 Gm. of crystallized ferric nitrate in 50 c.c. of distilled water. Add 2.5 c.c. of concentrated nitric acid and make up to 100 c.c. with distilled water.

*From the Department of Medicine, University of Colorado School of Medicine and Hospitals, Denver.

Received for publication, November 10, 1939.

3. Standards: Dissolve 0.0567 Gm. of potassium dichromate and 3.83 Gm. of cobalt chloride ($CoCl_2.6H_2O$) in 100 c.c. of distilled water. At times it may be more satisfactory to use larger quantities, that is, 0.567 Gm. of potassium dichromate and 38.3 Gm. of cobalt chloride in 1,000 c.c. of distilled water. The standard solution thus produced corresponds to 10 mg. of thiocyanate per 100 c.c. of serum. Other standards are made by proportional dilution of this standard solution; that is, the 5 mg. standard is obtained by mixing 5 c.c. of the 10 mg. standard and 5 c.c. of distilled water; the 4 mg. standard, by mixing 4 c.c. of the 10 mg. standard and 6 c.c. of distilled water, etc. Each standard is sealed in a comparator tube $\frac{7}{16}$ inch in diameter. If a comparator block with two rows is used, the standards absolutely essential are the 1, 2, 3, 4, 5, and 10 mg. standards. Since, however, the important therapeutic range is from 5 to 10 mg. per cent, it is possibly of some advantage to have all standards from 1 to 10. A blank of distilled water is essential.

4. Three or 4 comparator tubes calibrated for 1 and 2 c.c. Needless to say, the comparator tubes must be matched.

5. A comparator block with two rows. If only one row of the comparator block is used, the row next to the frosted glass is used. If both rows are used, the unknown solution must be balanced with the tube of distilled water.

6. A dropper which delivers 0.2 c.c. (for use with the ferric nitrate reagent).

7. A dropper which delivers 0.5 c.c. (for use with the trichloracetic acid solution).

8. One or 2 droppers calibrated to deliver 0.25, 0.5, 0.75, and 1 c.c. (for measuring serum).

9. One or 2 small funnels.

10. Filter paper to fit the funnels.

PROCEDURE

In one of the comparator tubes put water to the 2 c.c. mark. Add 0.5 c.c. of trichloracetic acid solution and 1 c.c. of serum and mix well. Filter into another comparator tube until 1 c.c. of clear filtrate is obtained. To this filtrate add 0.2 c.c. of ferric nitrate reagent. The color that forms is matched in the comparator with the standards. The reading is in milligrams per 100 c.c. of serum.

If 1 c.c. of serum is not obtainable, or if the reading with 1 c.c. of serum is over 19 mg. per cent, 0.5 or 0.25 c.c. of serum may be used and 0.5 or 0.75 c.c. of water, respectively, is added. The result must then, of course, be multiplied by 2 or 4 as the case may be.

REFERENCES

1. Barker, M. Herbert: The Blood Cyanates in the Treatment of Hypertension, J. A. M. A. 106: 762, 1936.
2. Griffith, John Q., and Lindauer, M. A.: Thiocyanate Therapy in Hypertension Including a New Micromethod for Determining Blood Thiocyanates, Am. Heart. J. 14: 710, 1937.
3. Massie, Edward, Ethridge, Clayton B., and O'Hare, James P.: Thiocyanate Therapy in Vascular Hypertension, New England J. Med. 219: 736, 1938.

METABOLISM OF ALLANTOIN*

Appearance in the Urine on Feeding and Related Experiments

Charles F. Schaffer, M.S., and Frederick R. Greenbaum, D.Sc.
Philadelphia, Pa.

ALLANTOIN is the chief end product of purine metabolism with most mammals. It is due to the uricolytic enzyme, uricase, which is found in the liver of these animals and converts uric acid into allantoin. According to Oikawa,[1] uricase, which he prepared from rabbit liver, acts best at 48° C. and is destroyed at 70° C. It is most active in an atmosphere of oxygen at a pH of 7.1 to 7.2, less active in an atmosphere of nitrogen, and practically inactive in carbon dioxide, carbon monoxide, and hydrogen atmospheres, respectively. Its activity is not effected by autolysis.

Borsook and Jeffreys[2] incubated surviving sections of isolated liver, kidney, spleen, diaphragm, and small intestine of the rat in Ringer's solution, and observed the formation of uric acid without any allantoin in every tissue except liver, with uric acid converted quantitatively to allantoin in the liver. Schuler and Reindel[3] presented evidence of its mode of formation. They oxidized uric acid by alkaline potassium permanganate and by oxygen in the presence of manganese dioxide and identified the intermediary product hydroxyacetylenediurioncarboxylic acid — HNCONHC(OH)NHCONHCCO$_2$H — which yields allantoin by loss of carbon dioxide. The similar behavior of this product in both enzymic and catalytic oxidation, and the identity of the trisilver salt isolated in both instances, are conclusive evidence that this product is the intermediate through which the breakdown of uric acid to allantoin occurs in the animal organism.

It is, therefore, reasonable to believe that such animals should completely absorb and subsequently excrete through the kidneys ingested solutions of allantoin without its undergoing molecular rearrangement. On the other hand, when the crystalline material is fed, a portion may fail to dissolve in the alimentary tract due to its comparatively low solubility, and consequently may appear in the feces.

The purpose of this report is to present the results obtained in a series of experiments designed to demonstrate the validity of the foregoing suppositions, together with certain ramifications encountered during the work.

*From the Biological and Research Laboratories of the National Drug Co., Philadelphia.
Presented before the Biological Division of the American Chemical Society Meeting, Boston, Mass., September 11-15, 1939.
Received for publication, November 10, 1939.

PRELIMINARY EXPERIMENTS

Prior to the actual feeding experiments the effect of the various gastrointestinal enzymes at their in vivo acidity or alkalinity, as the case may be, on standard solutions of allantoin was determined. Artificial gastric juice was prepared by digesting 10 Gm. of minced pigs' stomach mucosa with 50 c.c. of 0.4 per cent hydrochloric acid solution for twenty-four hours at 37° C., filtered and dialyzed to remove peptones and peptides. Intestinal enzymes were extracted from minced pigs' pancreas, using 1 per cent sodium bicarbonate in the proportion of 1 Gm. of organ to 3 c.c. of bicarbonate.

Standard allantoin solutions of varied concentrations were incubated in these enzymic extracts for a period of four hours at 37° C., and subsequently analyzed for allantoin. The results appear in Protocol I and obviously indicate no breaking of the allantoin.

PROTOCOL I

EFFECT OF GASTROINTESTINAL ENZYMES ON ALLANTOIN

ENZYMIC EXTRACT	ALLANTOIN ADDED MG. PER C.C.	ALLANTOIN RECOVERED MG. PER C.C.
Gastric	1.0	1.0
	1.5	1.5
	2.0	2.0
	2.5	2.5
Intestinal	1.0	1.0
	1.5	1.5
	2.0	2.0
	2.5	2.5

Rabbits were then fed definite amounts of allantoin. This was done by inserting a tube through the pharynx into the stomach, attaching a 10 c.c. syringe to the exposed end, placing the allantoin in the barrel of the syringe, and washing it through the tube with small amounts of water, with the assistance of slight pressure on the plunger. The animals were placed in metabolic cages and successive twenty-four-hour specimens of urine and feces were collected. The specimens of feces were triturated, extracted with water, and filtered. The filtrates were analyzed in conjunction with the urines for allantoin content, utilizing the method described by Larson.[4] No allantoin could be detected in the feces extracts in contrast to the urine analysis which gave tremendous recoveries of allantoin. The analytical data of these determinations are tabulated in Protocol II.

PROTOCOL II

RECOVERY OF ALLANTOIN AFTER INGESTION OF TARED AMOUNTS

RABBIT NO.	ALLANTOIN INGESTED	ALLANTOIN RECOVERED IN FECES	ALLANTOIN RECOVERED IN URINE	PER CENT RECOVERY
456	0.56 Gm.	None	1.40 Gm.	250
457	0.54 Gm.	None	1.76 Gm.	326
458	0.57 Gm.	None	1.75 Gm.	307
459	0.55 Gm.	None	1.84 Gm.	334

It was soon discovered, however, that allantoin ingestion greatly increased the excretion of creatine,[5] which in turn reduces the alkaline copper solution

utilized in this method for determining allantoin. Since creatine is not removed in the preliminary preparation of the specimen, it was the cause for the abnormally high results.

Protocol III shows this effect in tabulated form. In a large number of rabbit urines analyzed for both creatine and allantoin, allantoin is manifested chiefly in those urines containing creatine, and the concentration varies directly with the quantity of creatine present.

PROTOCOL III

EFFECT OF CREATINE ON ALLANTOIN DETERMINATION (LARSON)

URINE	CREATINE	ALLANTOIN
A1	3.2	5.0
A2	1.3	3.0
A4	0	0
A7	0	3.6
A8	9.3	7.4
B3	0	0
B5	4.7	6.7
B6	5.45	7.35
B7	0.5	0
B8	2.0	3.0

It was, therefore, necessary to develop a more specific method for the allantoin determination. The method finally adapted entirely eliminates the creatine present. It prepares the specimen according to the usual procedure, then utilizes the quantitative precipitating action of mercury salts on allantoin, producing mercuric allantoinate.

METHOD

The specimen is treated with an excess of 25 per cent phosphotungstic acid, followed by an excess of basic lead acetate and 5 per cent sulfuric acid solution. This removes most of the interfering substances. The entire procedure is carried out in the same pyrex tube. After the addition of each reagent the tube is rotated to insure thorough mixing and then centrifuged until clear. Two cubic centimeters of the supernatant liquid are transferred to another pyrex tube, neutralized to litmus, and the allantoin isolated by precipitating it with mercuric acetate-sodium acetate reagent. After standing one hour the mixture is centrifuged and the supernatant fluid is discarded. The precipitated mercuric allantoinate is then washed several times with water, rubbed thoroughly each time with the aid of a rubber policeman to insure penetration of the water, and centrifuged. All the wash water is discarded. The precipitate is finally dissolved in acetic acid and the nitrogen content is determined by micro-Kjeldahl analysis.

Reagents required: Phospho-24-tungstic acid.[6] This substance was prepared in our laboratories because the commercial grades are too impure for satisfactory results.

Basic lead acetate.[7]

Sulfuric acid solution, 5 per cent (by weight).

Sodium hydroxide, 5 per cent solution.

Mercuric acetate-sodium acetate reagent: Dissolve 1 Gm. of mercuric acetate and 10 Gm. of sodium acetate in water and make up to 100 c.c.

Glacial acetic acid, 20 per cent solution.
Litmus solution indicator.
Concentrated sulfuric acid.
Copper sulfate, 5 per cent solution.
Nessler's solution.[8]

PROCEDURE

Three and five-tenths grams of phosphotungstic acid are dissolved in water and diluted to 10 c.c. Two cubic centimeters of this solution are transferred to a pyrex tube and 2 c.c. of the specimen to be analyzed are added. Mix thoroughly and centrifuge. To the same tube add 2 c.c. of the basic lead acetate reagent, mix and centrifuge again. Finally, add 2 c.c. of the 5 per cent sulfuric acid solution and centrifuge until clear. Transfer 2 c.c. of the clear supernatant liquid to another pyrex tube and neutralize to litmus, using 5 per cent sodium hydroxide. After neutralization add 1 c.c. of the mercuric acetate-sodium acetate reagent, mix thoroughly, and set aside for one hour. Centrifuge and decant the supernatant fluid. Wash the precipitate several times with water, rubbing the precipitate each time with a rubber policeman attached to the end of a stirring rod, centrifuging and decanting. After the final decantation add 1 c.c. of 20 per cent glacial acetic acid to the precipitate and rub up until solution is affected. Then neutralize to litmus with a 5 per cent sodium hydroxide solution and make the micro-Kjeldahl[9] nitrogen determination as follows: Transfer the prepared sample to a Folin digestion tube which is conveniently graduated at the 35 and 50 c.c. marks. To this add 0.5 c.c. of concentrated sulfuric acid, 2 drops of a 5 per cent solution of copper sulfate, and several glass beads to prevent bumping. Then boil the mixture vigorously until the moisture is driven off and sulfur dioxide fumes fill the tube. Continue boiling the contents slowly until the fumes are driven off. The mixture should now appear pale blue green or yellowish in color. Allow the tube and contents to cool, dilute to the 35 c.c. mark with water, and add Nessler's solution to the 50 c.c. mark. Mix thoroughly and compare with an allantoin standard, standardized to contain 0.1 mg. of nitrogen per c.c., which has been digested in the same manner as the unknown. The unknown and standard should be nesslerized as nearly simultaneously as possible.

CALCULATIONS

$$X = \frac{(\text{Reading of standard})\ (\text{Concentration of standard})\ (\text{Dilution factor})}{(\text{Reading of unknown})\ (\%\ \text{nitrogen per mole of allantoin})}$$

The value for X will indicate the milligrams of allantoin per cubic centimeter of unknown.

REMARKS

The foregoing procedure was designed for use with urines, but has been used successfully with extracts of other material. The quantities of reagents cited are sufficient for the average normal urine specimens. However, should the material in question contain unusually high concentrations of proteins and their metabolic products, either the analysis should be diluted before performing or the proper balance of reagents should be determined. The former is obviously the better procedure.

The phosphotungstic acid precipitates the proteins while the addition of basic lead acetate removes the excess phosphotungstic acid and some other interfering substance. The subsequent addition of sulfuric acid precipitates the excess lead. The formation of a precipitate on neutralizing with sodium hydroxide indicates that the lead had not entirely been removed. On addition of acetic acid the precipitate may not entirely dissolve. This insoluble material is not mercuric allantoinate, but, at least a portion, is uric acid; therefore, remove by centrifuging and utilize the clear supernatant liquid only. A standard solution of allantoin is used as the comparison standard due to the difference in hue of the color developed on nesslerization.

EXPERIMENTAL

Rabbits were placed in metabolic cages and several twenty-four-hour specimens of urine were collected and analyzed to determine the daily normal excretion of allantoin. These animals were then fed weighed amounts of allantoin in the same manner as previously described, and several additional twenty-four-hour specimens of urine were collected and analyzed for allantoin. The tabulated results of these analyses appear in Protocol IV.

PROTOCOL IV

RECOVERY OF ALLANTOIN FED TO RABBITS

RABBIT NO.	ALLANTOIN FED	ALLANTOIN RECOVERED	PER CENT RECOVERY
	mg.	mg.	
775	536	628	117.16
776	500	486	97.20
777	550	333	60.54
778	550	1039	188.90
779	530	983	185.47
780	562	1048	186.48
781	538	477	88.66
782	530	979	184.70
783	590	884	149.83
784	510	1331	260.98
785	500	1133	226.60

With few exceptions the results indicate recoveries ranging from 100 per cent to 200 per cent, with one instance of 260 per cent recovery. The excess over that normally present and that ingested seems to indicate that possibly some organ retains the substance which, during periods of prolonged fasting, can be utilized in the resynthesis of cellular products, and the forced feeding of amounts in the concentrations given stimulates that organ to the point of causing this allantoin to be excreted. To obtain more information on this theory, analyses were made on aqueous extracts of macerated kidneys and livers of both normal and allantoin fed rabbits. The results for the liver analysis are recorded in Protocol V.

These results appear to indicate the validity of the afore-mentioned theory and point to the liver as the organ retaining a concentration, for in normal liver a concentration of 0.44 mg. to 0.60 mg. of allantoin per gram of organ is found in comparison to 0.09 mg. to 0.35 mg. of allantoin per gram of organ in animals that were fed 0.5 Gm. of the substance eight hours previously. On the other hand, normal kidneys show the presence of allantoin to the extent of 0.16 mg.

PROTOCOL V

EFFECT OF ALLANTOIN INGESTION ON ALLANTOIN CONTENT OF LIVER

RABBIT NO.	MG. ALLANTOIN PER GM. OF ORGAN	
	NO ALLANTOIN FED	INGESTED 8 HOURS PREVIOUSLY
991	0.44	
992	0.51	
993	0.60	
994	0.49	
995		0.33
996		0.13
997		0.35
998		0.09
Average	0.51	0.225

per gram of organ, whereas the animals fed allantoin show a content of 1.17 mg. per gram of organ.

Effect of Fasting on Allantoin Content of Liver.—Three groups of two rabbits each were placed on a starvation diet to determine the effect of inanition on the allantoin content of the liver. One group was sacrificed at the end of three days, another in five days, and the third seven days after inanition.

The results represent the average values for the analysis made on the pooled material of each group. The livers and kidneys were macerated and extracted with twice their weight of water at 40° C. After forty-eight hours' extraction the material was filtered and the filtrate was analyzed for allantoin, utilizing the procedure already outlined.

The analysis of the urines excreted during this period showed a gradual but definite increase of allantoin excretion, paralleling the analytical results for allantoin in kidneys which increased with each successive inanition period.

Results of the liver and kidney analysis appear in Protocol VI.

PROTOCOL VI

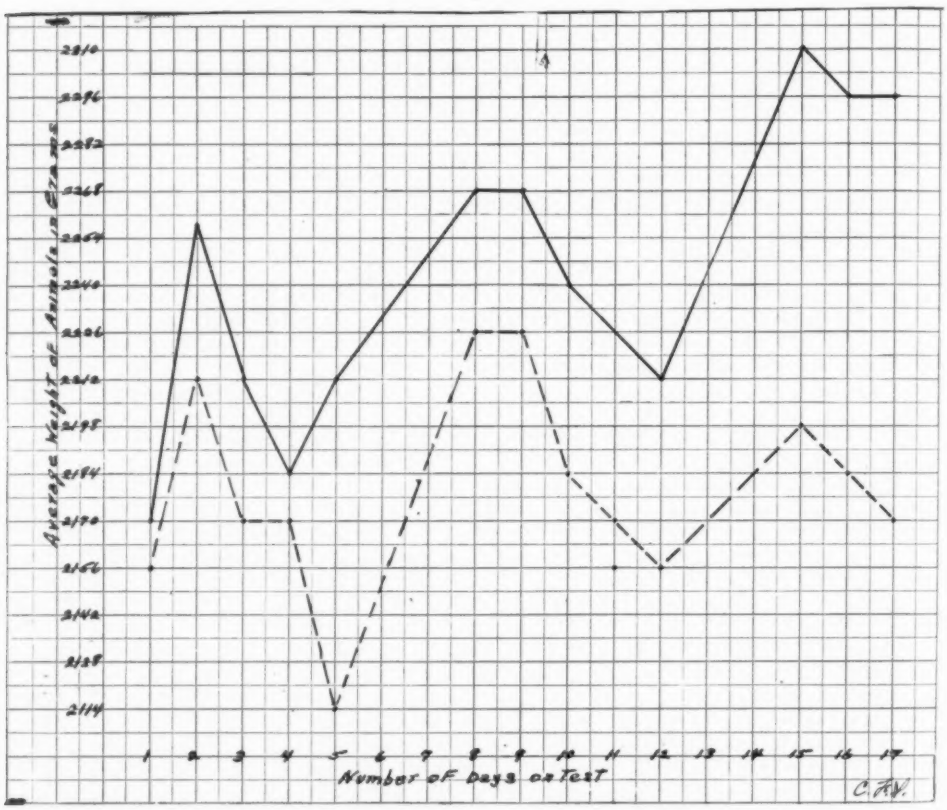
EFFECT OF FASTING ON ALLANTOIN CONTENT OF LIVER AND KIDNEY

PERIOD OF FAST	MG. ALLANTOIN PER GM. OF ORGAN	
	IN LIVER	IN KIDNEY
3 days	0.61	0.21
5 days	0.32	0.35
7 days	1.0	1.16

The drop in the allantoin content of liver between the first and second inanition periods would tend to support the theory that allantoin can be resynthesized into cellular products during time of fasting. On the other hand, the results obtained after seven days of inanition appear to indicate a tremendous increase of uricolytic enzyme stimulation. According to Oikawa,[10] who reported on the effect of inanition upon the uricolytic activity of the liver extract, during starvation experiments for twenty-four hours with the rabbit, no effect on uricolytic activity was noted in the first week, but some increase was obvious in a longer period of time.

Effect of Allantoin Ingestion on Body Weight.—The tremendous increase of creatine excretion prompted experiments to determine whether this creatine represented cellular loss and probably led to eventual depletion of cellular creatine. This was done by feeding various amounts of allantoin to rabbits

daily and observing changes occurring in body weight. Graphs 1 and 2 show the effect of ingested allantoin on body weight of rabbits. Each curve represents the average weight of two rabbits. In Graph 1 the test animals were fed 100 mg. per kilogram body weight per day. As shown by this curve the maximum and minimum weight fluctuation over a period of sixteen days approximates a straight line, indicating a remarkably constant body weight. However, there is definite inhibition of weight increase in these animals.

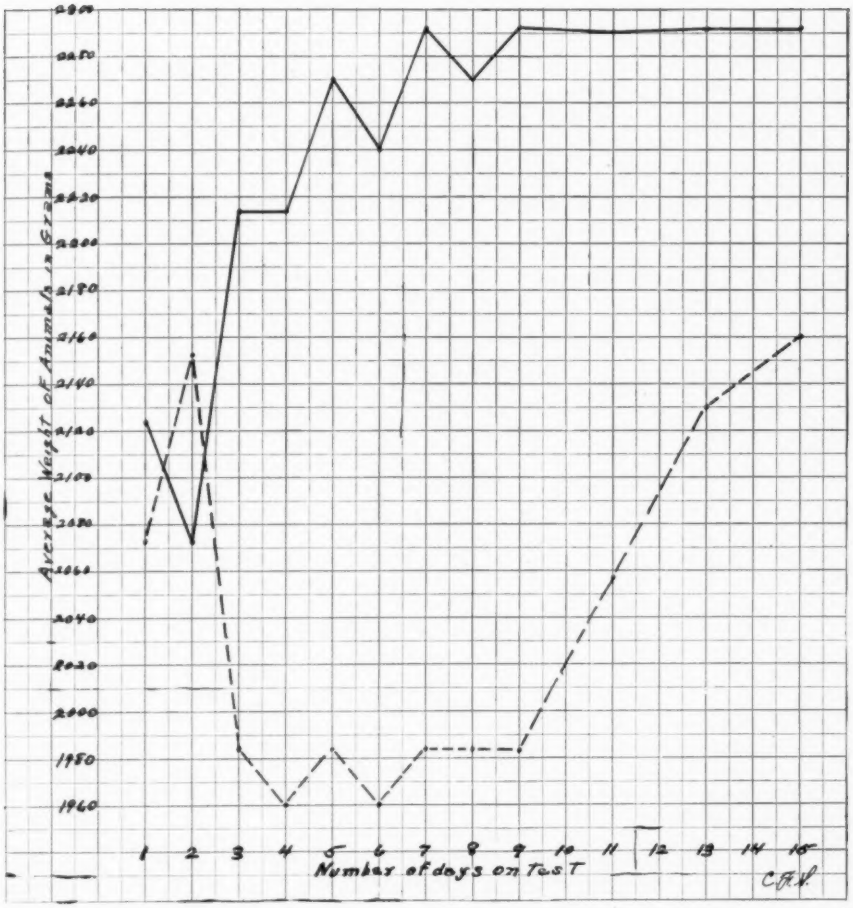


Graph 1.— ———— Control animals.
—— —— Animals fed 100 mg. per kg. body weight per day.

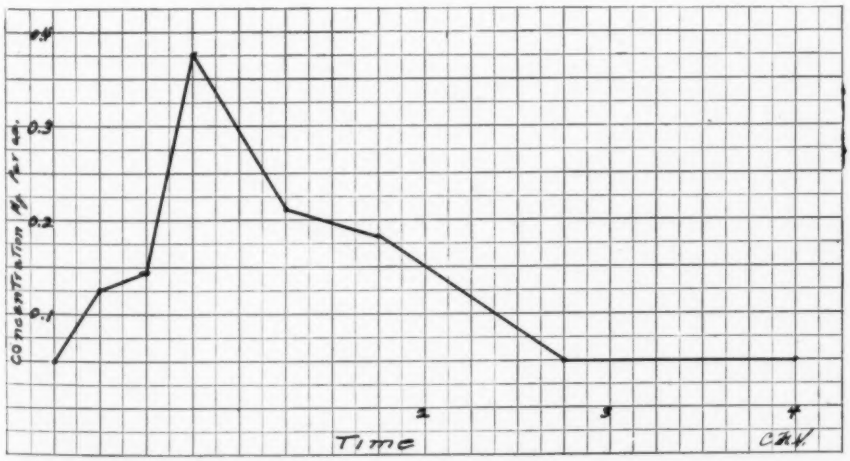
Graph 2 shows the effect of feeding 250 mg. per kilogram body weight per day. Here, too, the curves represent the average weight of two rabbits. The curve representing the test animals shows a decided decrease in weight throughout the test period in contrast to the curve representing the control animals. However, recovery of lost body weight during the test period is rapidly replaced on removal of the allantoin from the diet, as is shown by that portion of the curve lying between the ninth and thirteenth day period.

The feeding of amounts less than 100 mg. per kilogram body weight had no effect on normal body weight increase.

After completion of the work reported here, there appeared the publication of Beard and Pizzolato[11] on the effect of parenteral injection of purines, methylated purines, and various drugs upon creatine-creatinine metabolism in the



Graph 2.— ———— Control animals.
—— —— Animals fed 500 mg. per kg. allantoin per day.



Graph 3.—Maximum concentration of allantoin in circulation on intravenous injections.

muscles of the rat. They reported that adenine, xanthine, hypoxanthine, uric acid, and allantoin gave increases in muscle creatine as well as large increases in creatine excretion, showing that the urinary creatine did not originate from that in the muscles. They concluded that these substances stimulated creatine formation and excretion of the body in a direct proportion to the number of methyl groups, up to 3, present in the supplement injected.

Maximum Concentration of Allantoin in Circulation on Intravenous Injections.—Rabbits were given intravenous injections of allantoin to determine the interval in which the maximum concentration of allantoin appears in the circulation, the amount recovered at that time, and the time required for the injected allantoin to disappear entirely from the blood stream. Forty milligrams of allantoin were injected into each animal and bleedings were made (heart puncture) at fifteen-minute intervals. As shown in Graph 3, forty-five minutes after the injection the maximum concentration of allantoin was manifest. At this point 38.54 mg., or 96.36 per cent, of allantoin were recovered. At the end of two hours and forty-five minutes the blood concentration had dropped to normal.

SUMMARY

1. The method described by Larson[4] for the determination of allantoin in urine cannot be used on urines containing creatine. Due to the fact that allantoin injection causes the excretion of large amounts of creatine, it was necessary to develop a procedure which eliminated the creatine entirely. This was done by precipitating the allantoin as the mercuric allantoinate.

2. Allantoin ingested by rabbits is completely absorbed and subsequently excreted by the kidneys. No allantoin could be detected in the intestinal excreta.

3. Allantoin recovery experiments after ingestion of tared amounts by rabbits, indicate quite an excess over that ingested and normally present. This excess was traced, partially at least, to the liver. This organ loses its normal concentration of allantoin when that substance is fed. Experiments on the explanation of this phenomenon are in progress.

4. Effect on body weight of animals fed allantoin daily to determine the probable origin of the increased creatine output, showed a decided loss in weight when amounts above 250 mg. per kilogram of body weight were fed. This would seem to indicate that the excess creatine was derived from cellular creatine.

5. Allantoin injected intravenously reaches its maximum concentration forty-five minutes after injection. At this point 96.36 per cent can be recovered. At the end of two hours and forty-five minutes the blood concentration of allantoin has dropped to normal.

REFERENCES

1. Oikawa, R.: Uricase. I. Sei-I-Kai M. J. **52**: 1, 1933. (English Abstract 1.) C. A. 28: 7280, 1934.
2. Borsook, H., and Jeffreys, C.: Nitrogen Metabolism of the Isolated Tissues of the Rat, J. Biol. Chem. **110**: 495, 1935.
3. Schuler, W., and Reindel, W.: Uricolysis III, Ztschr. f. physiol. Chem. **215**: 258, 1933.
4. Larson, H.: A Colorimetric Method for the Determination of Allantoin, J. Biol. Chem. **94**: 727, 1932.

5. Schaffer, C.: A New and Direct Method for the Determination of Creatine. I. Preliminary Report, J. Am. Chem. Soc. **60**: 2001, 1938.
6. Wu, H.: A Contribution to the Chemistry of Phosphomolybdic and Phosphotungstic Acids and Allied Substances, J. Biol. Chem. **43**: 197, 1920.
7. Hawk and Bergeim: Physiological Chemistry, ed. 11, Philadelphia, 1937, P. Blakiston's Son & Co.
8. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38**: 81, 1919.
9. Folin, O., and Wu, H.: A System of Blood Analysis, J. Biol. Chem. **38**: 81, 1919.
10. Oikawa, R.: Uricase. IV. Effect of Inanition Upon the Uricolytic Activity of the Liver Extract **53**: 43, 1933 (English Abstract 3). C. A. **28**: 7280, 1934.
11. Beard, H. O., and Pizzolato, P.: The Effect of Parenteral Injection of Purines, Methylated Purines, and Various Drugs Upon Creatin-Creatinine Metabolism, J. Pharmacol. & Exper. Therap. **63**: 306, 1938.

TECHNIQUES FOR THE PREPARATION AND CARE OF PANCREATIC FISTULAS IN DOGS*

V. Brown Scott, Ph.D., M.D., Bloomington, Ind.

DURING the past two years we have prepared and cared for over 250 pancreatic fistula dogs. This experience has enabled us to establish fistulas suitable for *extended* observations. It is believed that an account of the techniques for the preparation and care of these fistulas, which have proved most satisfactory in this laboratory, would be of assistance to those attempting such preparations for the first time.

We have prepared fistulas according to the methods of Babkin,[1] Dragstedt,[2] Elman and McCaughan,[3] and Inlow.[4] No attempt will be made to review these methods; the reader is referred to Babkin,[5] and Boldyreff.[6] The most satisfactory fistulas have proved to be those of Dragstedt and Inlow. The operations and the postoperative animal care, as modified by us, will be described in detail sufficient to facilitate such preparation. All operations are performed with rigorous asepsis and full surgical anesthesia.

THE DRAGSTEDT FISTULA

In the Dragstedt fistula the pancreatic juice is conducted into a small duodenal pouch through the *undisturbed* major pancreatic duct; a cannula in this pouch conducts the secretion to a football bladder suspended beneath the dog's abdomen. (In Dragstedt's published method the duodenal pouch received *all* the pancreatic ducts. Fistulas of the major pancreatic duct *only* have been used in his laboratory for years.[7]) The cannula (Fig. 1) is made of "18-8" stainless steel and satisfactorily replaces the gold plated one of Dragstedt.

Operation.—An 18 to 20 kg. female dog is anesthetized with ether following preoperative morphine and atropine. The abdomen is opened by a 10 cm. incision, starting just below the xiphoid and ending 3 cm. to the right of the midline. The duodenum is grasped with moist sponges and delivered into the wound. The antimesenteric duodenal border is pulled to the right (operator

*From the Department of Physiology, Indiana University School of Medicine.
Received for publication, November 15, 1939.

on right side of animal), bringing the left side of the gut uppermost. The major pancreatic duct joins the duodenum 3 to 4 cm. rostral to the most caudal application of the pancreas to the duodenum. The common bile duct, which enters the bowel rostral to the major pancreatic duct, is located at its point of entrance into the bowel.

The aseptic end-to-end anastomosis, which has proved most satisfactory in this laboratory, has been developed by Martzloff and Burget[8] (1931). Long Carmalt artery forceps are ground on one side to reduce the blade width to 3 mm.; this facilitates the inversion of a *small* diaphragm.

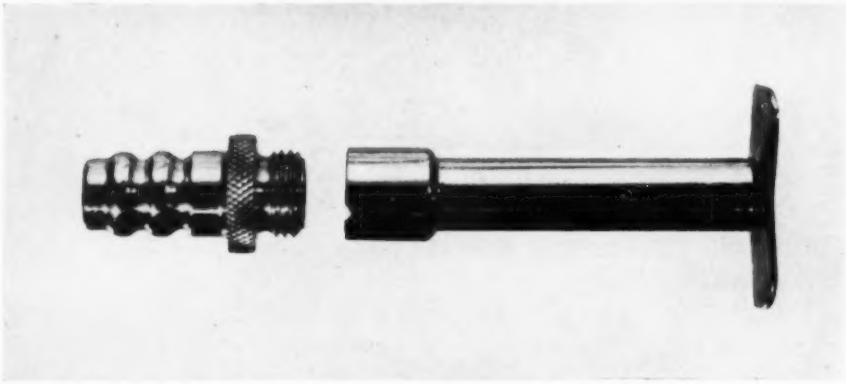


Fig. 1.—Photograph of a 2 inch "18-8" stainless steel cannula with a "screw tip."

Fifteen centimeters of bowel below the common bile duct are packed off outside the abdomen to reduce peritoneal soilage. Midway between the common bile duct and the major pancreatic duct, Martzloff forceps are placed across the bowel. The "ground" side of the forceps is faced rostrad (handle away from the operator). Another forceps (handle toward operator and "ground" side caudad) is placed across the bowel just below and touching the upper forceps. Both forceps are closed as completely as possible. About 8 cm. caudad on the bowel the above procedure is duplicated, except that the distal forceps is set with the "ground" surface caudad (handle away from the operator). The bowel between the upper and lower pairs of forceps is severed flush with the forcep's blades; the cut surfaces are treated with 95 per cent phenol followed by dilute alcohol.

A 1 cm. incision is made midway between the forceps on the antimesenteric border of the duodenal pouch segment. The caudal forceps is removed and the "conical tip" of the cannula (see Fig. 2) is introduced into the pouch lumen and brought out through the antimesenteric wound. The caudal end of the pouch is clamped shut with Martzloff forceps, excess tissue is removed, and the cut ends of the bowel are recauterized with phenol. Both ends of the pouch are closed by oversewing the forceps (Parker-Kerr technique[9]), using No. 1 silk suture on a straight intestinal needle. A purse-string suture is placed in the pouch about the cannula; the wound edges are invaginated and the suture is tied just tightly enough to prevent pericannular leakage.

End-to-end anastomosis of the severed ends of the proximal and distal duodenum is now begun. The Martzloff forceps are turned by the assistant, so

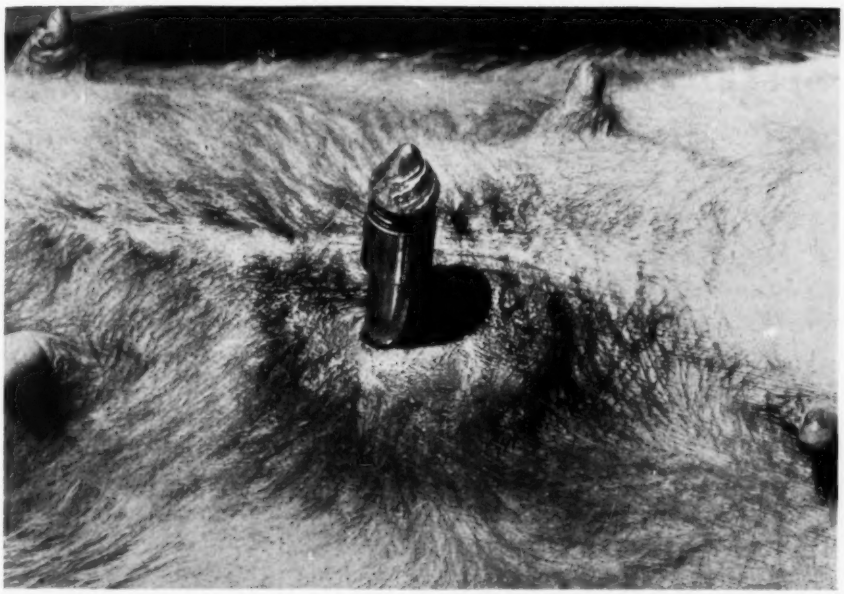


Fig. 2.—Photograph of the abdomen of a Dragstedt fistula dog. The "conical tip" has been *partially* screwed into the stainless steel cannula for illustrative purposes.

Fig. 3.—Photograph of the abdomen of an Inlow fistula dog. Note that the pancreatic duct orifice occupies the *center* of the picture. At the time the photograph was taken this dog had secreted pancreatic juice continuously for a period of more than six months.

that the "ground" surfaces face the operator and the *under* surfaces of the severed ends of the duodenum are adjacent. A continuous Connell suture is laid between these duodenal surfaces. A No. 1 silk suture, on a fine curved intestinal needle, is begun on the mesenteric border of the proximal bowel close to the forceps and ended at the antimesenteric border of the distal bowel segment. The Martzloff forceps are rotated to bring the "unground" surfaces of the forceps together and the cut ends of the bowel directly opposite. Another Connell suture is placed across the forceps, starting on the mesenteric border of the distal bowel close to the caudal forceps and finishing above the rostral forceps at the bowel's antimesenteric border. The antimesenteric sutures are grasped in one hand, the mesenteric ones in the other, and moderate tension is maintained while the forceps are removed by the assistant. The cut ends of the bowel are drawn together snugly and sutures are tied after inspection shows satisfactory bowel approximation. (Sutures should not be tied too tightly.) The anastomosis is manipulated to establish luminal patency and examined for leaks. A row of interrupted silk Halsted sutures is placed to reinforce the anastomosis. (The anastomosis must be tight because leakage is followed by peritonitis.)

The pouch and base of the cannula are draped carefully with omentum. Four interrupted linen sutures are passed through the pouch wall, 1 cm. in each direction from the cannula. A midline stab wound (opposite the junction of the middle and lower thirds of the abdominal wound) is made which is just large enough to admit the "conical tip" of the cannula that is brought through it. The linen sutures are placed through the abdominal wall about the cannula with a Reverdin needle; the sutures are tied tightly enough to hold the pouch against the peritoneum. This procedure,[10] which prevents intra-abdominal leakage and subsequent peritonitis by holding the pouch to the peritoneum, is a most effective one.

After washing the hands thoroughly, the abdomen is closed with clean instruments and catgut suture. The skin is sutured with interrupted linen and the wound sponged with tincture of iodine. No bandage is applied to the wound.

Postoperative Treatment.—For the first three to five days the animals are given no food or water. Intravenous Ringer's solution (100 c.c. per kg. body weight) is administered daily during this period. A padded high metal collar[11] prevents the animal from disturbing the cannula or the wound. As soon as pancreatic secretion appears, the dogs are placed in cages having wire bottoms in order to avert the development of pedal ulcers; such ulcers develop from prolonged contact with *active* pancreatic juice.

A soft diet is given about the fifth postoperative day; the pouch-anchoring sutures are removed at this time. Subsequently the diet consists of dog biscuits and milk. Sodium chloride (0.5 Gm. per kg. body weight per day) is added to the drinking water in order to replace the electrolyte lost by the dog through pancreatic secretion.

Collection of pancreatic juice, by means of a football bladder carried in an apronlike bag tied about the dog's abdomen, is initiated about the sixth postoperative day.

Postoperative complications are peritonitis and obstruction of the bowel. Peritonitis may result from a leak in the duodenal anastomosis or rupture of the duodenal pouch. Intestinal obstruction, which may occur in an animal possessing an intestine with a small lumen, is an unusual complication. The side-to-side anastomosis which would eliminate such obstruction, cannot be used for the high duodenal anastomosis.

THE INLOW FISTULA

Of the various pancreatic fistulas in which the major pancreatic duct is transplanted to the anterior abdominal wall, this preparation has proved most satisfactory in our hands. The two-stage operation is far superior to the single-stage one because of the lower postoperative mortality. The operation is superior to the method of Pavlov since the Inlow fistula can be prepared easily. The Inlow method has been modified in this laboratory to simplify the operative procedure and to secure a higher percentage of satisfactory fistulas.

Operation. First stage.—A 10 to 20 kg. dog is anesthetized with ether. A straight 10 cm. right rectus incision is made through the skin and subcutaneous tissue, starting just below the costal border, 2 cm. lateral to the midline. The subcutaneous tissue is separated from the fascia laterally to form a pocket which will receive the transplanted duodenum. The abdominal incision is completed along the original line, the duodenum is delivered into the wound, and the major pancreatic duct is located *vide supra*. The pancreas is freed from the duodenum 2.5 cm. above and below the duct after careful ligature of all blood vessels.

The transplantation of the duodenum beneath the abdominal skin is accomplished by closure of the fascia, muscle, and peritoneum, about the gut and pancreas adjacent to the pancreatic duct by four No. 2 chromic catgut-mattress sutures. The most rostral suture is passed between the bowel and pancreas about 3 cm. above the duct (which should lie about the center of the wound). The lowest suture is placed between the gut and the blood vessels at a region about 3 cm. below the duct. These sutures are placed so that the wound will fit snugly about the bowel where the latter enters the abdomen. The intermediate mattress sutures are placed *straight* through the pancreas near its free border at levels, just above and below the duct. This ensures the exteriorization of sufficient pancreas. (In placing the sutures the blunt end of the needle proceeds first to avoid blood vessel rupture.) The mattress sutures, with the exception of those passing directly through the pancreas, are tied tightly.

The duodenum is rotated to the right as far as possible and is fixed to the fascia beneath the lateral skin flap (catgut sutures between the rectus fascia and intestinal submucosa). This holds the gut and pancreas firmly and places the pancreatic duct in a *superficial* position at least 2.5 cm. lateral to the wound margin. After all hemorrhage has been controlled the subcutaneous tissues and skin are closed over the transplanted bowel with catgut and linen sutures.

Second stage.—After a four-week interval (to reduce hemorrhage at second operation) the transplantation of the major pancreatic duct is performed

under ether anesthesia and careful asepsis. The middle 6 cm. of the old skin wound is reincised, and the pancreatic duct and adjacent pancreas are located by cautious dissection. After careful mobilization and dissection of the duct to secure maximum length, the duct is severed partially at its intestinal junction. Fine silk, on a Carrel artery needle, is passed through the free lip of the duct. The duct is now completely severed from the bowel, and the defect in the latter is closed with a short continuous Lembert suture. Three additional sutures are placed through the end of the duct. A small stab wound, large enough to accommodate the duct, is made in the skin directly over the base of the duct (about 2.5 cm. from the edge of the incision). In order to reduce the tension upon the sutures between the duct and the skin, catgut sutures are placed between the duodenum and the cutaneous tissues on either side of the stab wound. *This anchors the skin firmly to the bowel.* The pancreatic duct is delivered easily through the stab wound and anchored *loosely* to the skin by the silk sutures. The original incision is now closed in the usual manner with catgut and linen.

Postoperative treatment.—The dog is placed in a cage having a wire bottom and his neck is fitted with a suitable padded collar to prevent his disturbing the wound. The dog can be fed as soon as his appetite returns. The diet consists of dog biscuits; sodium chloride is added to the drinking water. The duct sutures are removed about the fourth postoperative day.

Alkali dermatitis often develops in animals which secrete profusely. This is treated best by daily baths and applications of potassium permanganate 1:1,000. The chowchow, which has an alkali-resistant skin, requires less attention in this respect than other breeds (Fig. 3).

The pancreatic duct orifice is probed daily to avoid closure of the duct which occasionally occurs if this precaution is overlooked.

COMMENT

The Dragstedt fistula is an excellent preparation for those experiments in which pure pancreatic juice is not required. This fistula has the advantages that the secretion is collected over the entire day; the animals remain dry and hence require little postoperative care; the normal duct-intestinal relationships are undisturbed. The chief disadvantages of this fistula lie in the rather high incidence of postoperative peritonitis and the admixture of succus entericus, which contaminates the pancreatic juice in unknown quantities.

The Inlow type yields pure pancreatic juice which is collected, during an experiment, by a glass cannula introduced into the transplanted duct or by a glass funnel cemented by collodion to the abdomen adjacent to the duct. The fistula's chief disadvantages are alkali dermatitis as a result of profuse secretion and sensitive abdominal skin, closure of the duct through failure to probe it daily and inability to collect easily the entire daily secretion.

Observations of reasonable duration are made in both types of fistula on dogs which are trained to lie quietly upon a padded table. Drop-by-drop secretion is recorded by an automatic drop counter connected to the fistula. Extended observations are made upon animals placed in stocks.

REFERENCES

1. Babkin, B. P.: Die Aussere Sekretion der Verdauungsdrüsen, Berlin, 1928, Julius Springer, p. 459.
2. Dragstedt, L. R., Montgomery, M. L., Ellis, J. C.: A New Type of Pancreatic Fistula, Proc. Soc. Exper. Biol. & Med. **28**: 109, 1930.
3. Elman, R., and McCaughan, J. M.: On the Collection of the Entire External Secretion of the Pancreas Under Sterile Conditions and the Fatal Effect of the Total Loss of Pancreatic Juice, J. Exper. Med. **45**: 561, 1927.
4. Inlow, W. D.: A Technic for the Establishment of a Permanent Pancreatic Fistula With the Secretion of Inactive Proteolytic Ferment, J. Lab. & Clin. Med. **7**: 86, 1921.
5. Babkin, B. P.: Die Aussere Sekretion der Verdauungsdrüsen, Berlin, 1928, Julius Springer, p. 456.
6. Boldyreff, W. N., and Martin, W. F.: The Operation for and the Importance of a Permanent Closeable Pancreatic Fistula, Tohoku J. Exper. Med. **35**: 144, 1939.
7. Dragstedt, L. R.: Personal communication, 1936.
8. Martzloff, K. H., and Burget, G. F.: The Closed Intestinal Loop. III. Aseptic End-to-End Intestinal Anastomosis and a Method for Making a Closed Intestinal Loop Suitable for Physiologic Studies, Arch. Surg. **23**: 26, 1931.
9. Markowitz, J.: Textbook of Experimental Surgery, Baltimore, 1937, William Wood & Co., p. 86.
10. Markowitz, J.: Textbook of Experimental Surgery, Baltimore, 1937, William Wood & Co., p. 136.
11. Mann, F. C.: Personal communication, 1938.

CONTINUOUS VACUUM DISTILLATION*

Martin Burger, M.S., New York, N. Y.

THE isolation of bacterial specific substances from broth culture media, like many other procedures in organic chemistry, requires concentration of a large bulk to a small volume by means of vacuum distillation. It has become advantageous, therefore, to employ some time-saving modification in the usual apparatus.[1, 2]

A very inexpensive and efficient assembly is herein described and diagrammed.

USE OF APPARATUS

After the system has been evacuated and the receiver *B* simultaneously cooled, the liquid from a stock bottle is transferred to the distillation flask by means of a siphon tube connected to the side arm *H, G, F*. Cocks *H* and *G* are opened, and when the desired amount has been transferred, they are closed. The siphon tube is then disconnected and the capillary tipped *I* is replaced. If frothing occurs, it may be controlled by manipulating *H* and *G* as described below. The distillation is now conducted until removal of the distillate is required. For removal, the three-way stopcock *D* is turned to evacuate catch flask *E*, and the screw clamp *J* is opened. By momentarily admitting air into the distillation flask via screw clamp *C* or cocks *H* and *G*, the distillate will be drawn through tube *A* into the catch flask. After drawing off the distillate, *J* is closed and *D* is turned to continue the distillation.

*From the William Hallock Park Laboratory, Bureau of Laboratories, Department of Health, New York.

Received for publication, November 18, 1939.

By this procedure distillation need not be interrupted. It is unnecessary to disconnect the vacuum pump and separate the receiver in order to pour off the distillate; reconnect and reseal broken points of attachment and start evacuating again. These are all time-consuming elements.

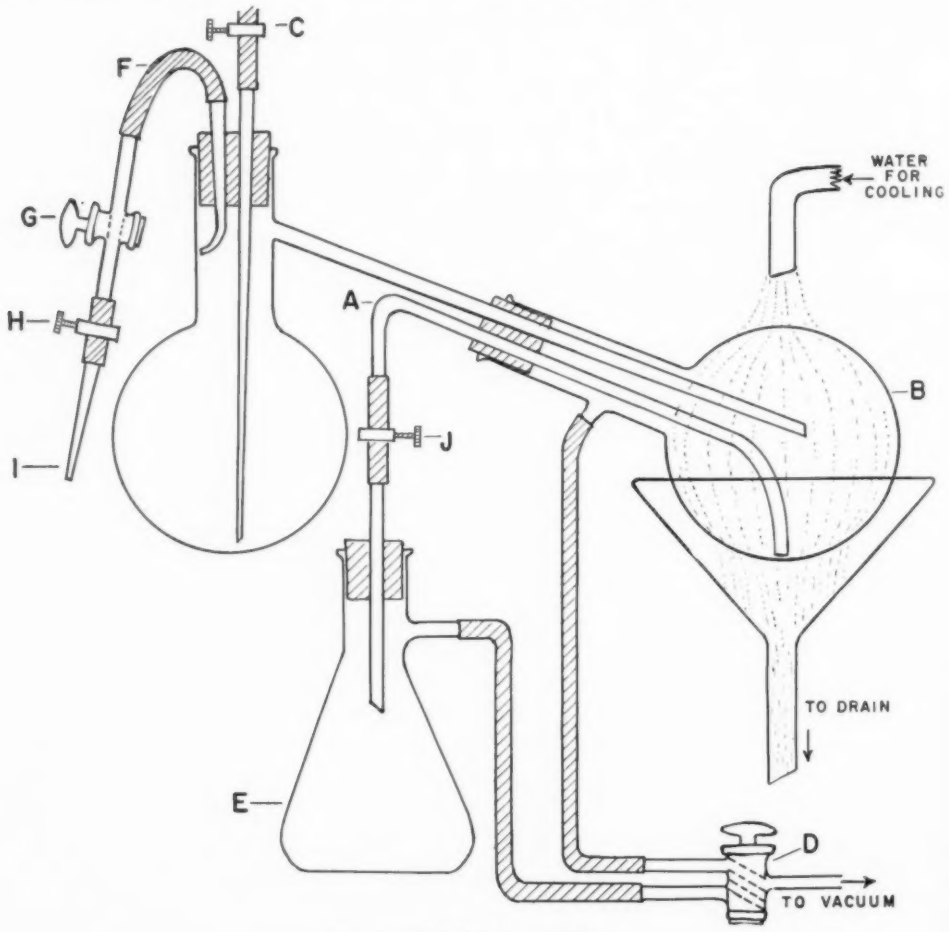


Fig. 1.—Distillation assembly.

DESCRIPTION OF APPARATUS

As shown in Fig. 1, a distilling flask is inserted into one of the holes of a two-hole rubber stopper, the usual capillary tipped glass tube. At the top of this is attached a rubber tube with a screw clamp *C*. The other hole is fitted with a bent glass tube *F* connected to a glass stopcock *G*. A piece of glass tubing drawn to a capillary or fire polished to a fine orifice *I* is connected to *G* by means of a rubber tube. Affixed to the latter is a screw clamp *H*. This modification may be used for two purposes: (1) As a froth controlling device[3] by using stopcock *G* as a coarse adjustment "valve" and screw clamp *H* as a fine adjustment. (The use of octyl or other higher alcohols as antifroth agents is eliminated.) By manipulating these "valves," the incoming air pressure prevents frothing over of the material into the receiver *B*. A little practice is

required to obtain optimum adjustment. While frothing is controlled, the distillation is evident by the appearance of a cloud formed in the receiver. The cold water or ice surrounding *B*, however, causes condensation of the cloud. (2) As an intake tube, the capillary *I* is removed and a siphon tube is attached. The other end of the siphon extends into the stock bottle containing the liquid to be distilled. For this purpose, it is advisable to fit the bent tube *F*, so that the end projecting into the distillation flask just touches the side of the neck. The incoming liquid then strikes the neck and flows smoothly down into the bowl of the flask, thereby preventing any additional froth. When the flask is filled to the desired level, the siphon tube is disconnected and the capillary *I* is replaced. Any frothing which occurs may be controlled.

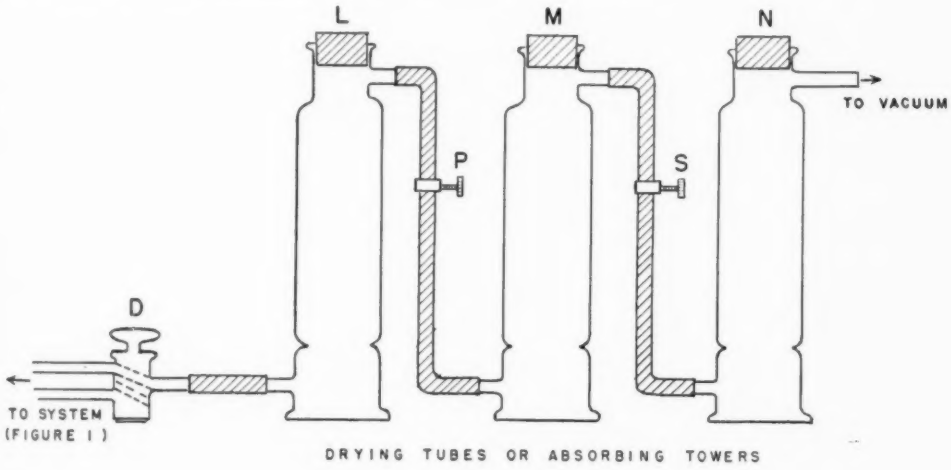


Fig. 2.

The condenser and receiver *B* also have a two-hole rubber stopper. One hole is for the side arm of the distillation flask which extends into *B*. Into the other hole is inserted a piece of glass tubing *A*. That end of the latter which projects into the receiver is bent, so that it just reaches the bottom of the distillate. At the other end is attached a length of rubber tube to which is affixed screw clamp *J*. From this, a piece of glass tubing extends into a one-hole rubber stopper in the mouth of catch flask *E*.

One of the parallel ends of a three-way stopcock *D* is connected to the catch flask, the other to the side arm of the receiver. The single end is connected to the drying tubes or absorbing towers and the vacuum pump.

A further improvement in the setup is obtained by arranging the drying tubes or absorbing towers, as shown in Fig. 2. When the first tube *L* becomes saturated with moisture and requires refilling with desiccant, it is merely necessary to close stopcock *D* and screw clamp *P*. Remove *L* and attach the rubber tube which connected the drying tubes *L* and *M* to stopcock *D*. *P* and *D* are then opened. *L* is recharged with desiccant and put back into operation again in its original position.

For maintaining sterile conditions, as in the distillation or concentration of biological products, e.g., sera, culture media, hormones, vitamins, etc., it is

necessary to connect a train of two or three bottles containing phenol or other bactericidal solution to *C* and *H*, the two points where air is admitted. The incoming air thereby is washed free from contaminants before entering the system. When the bactericide is already present in the liquid to be distilled, it is unnecessary to use the trains.

SUMMARY

A continuous vacuum distillation assembly is described and diagrammed.

REFERENCES

1. Kummer, R.: A New Vacuum Distillation Apparatus, Chem. Fabr. **1**: 7, 1928.
2. Semenov, V. M.: A Continuous Vacuum Distillation, J. Chem. Ind. Moscow **5**: 722, 1928.
3. Burger, M.: An Accessory "Valve" for Control of Frothing in Distillation, Chem-Analyst. To be published.

THE MAZZINI TEST: A GREATER AID IN THE SERODIAGNOSIS OF SYPHILIS*

A. W. Ratcliffe, M.D., Hammond, Ind.

PRELIMINARY STUDIES

IN DEVELOPING his recently published flocculation test for syphilis, Mazzini[1] collected such information as could be obtained concerning those cases showing definite discrepancy in the results of the various tests employed in the Serological Laboratory of the Indiana State Board of Health. I have followed his work with great interest, and during the past several months have had the opportunity to add a few cases to the material he has collected and to employ his test in its first use in actual practice. It is with his permission that the material he collected has been analyzed and is herewith presented. The information was collected by means of questionnaires sent to the physicians in charge of the cases that showed definite discrepancies. As has already been stated,[1] the Mazzini test early demonstrated a greater degree of sensitivity in general than did the other tests we were using routinely. This was so apparent that in the cases of partial positives a slightly stronger result with this test was soon regarded as the usual finding and not as indication for a questionnaire. Questionnaires were not sent on specimens received marked for "treatment check."

In Table I are presented 502 cases in which there was discrepancy between the results of the various serologic tests. The cases classified as syphilitic are undoubtedly so according to the available information. Usually a definite statement indicating previous diagnosis of, or previous treatment for, syphilis was accepted as sufficient for syphilitic classification. In one or two cases sufficient information was given to question such a statement; these cases have been classified as doubtful with suggestive data.

*From the Bureau of Bacteriology and Pathology and the Lake County Survey Laboratory of the Indiana State Board of Health.

Received for publication, December 7, 1939.

In general, the cases classed as doubtful with suggestive data are cases which might be regarded as diagnostic problems, with syphilis as a definite possibility. We have been "liberal minded" to the extent of considering that syphilis in a marital partner, congenital syphilis in a sibling, or a history of abortion or prostitution was suggestive of syphilis.

The cases listed as doubtful without suggestive data for the most part are so classed because of inadequate information. For example, a terse reply to the effect that the patient gives no history of syphilis is not sufficient evidence upon which to rule out the infection. The cases classed as nonsyphilitic are those rare cases in which sufficient information was obtained to exclude syphilis.

Table I is set up with the Mazzini test result as the basis of classification in the vertical column and the probable diagnosis in the horizontal spread. For example, 157 cases showed a 4-plus Mazzini reaction; of these, 97 were syphilitic, 41 were doubtful with suggestive data, and 19 were doubtful without suggestive data. Of the 157 cases, 18 gave negative Kline reactions; 81 gave negative reactions to the modified Kahn test; and 141 gave negative reactions with the complement fixation test. Not only did these negatives occur but also many of the other specimens gave only partial positives with each of the three tests; necessary condensation of the original table makes it impossible to show this here.

TABLE I

CASES SELECTED BY MAZZINI SHOWING DISCREPANCIES IN THE RESULTS OF VARIOUS SEROLOGIC TESTS FOR SYPHILIS. (Selected From Approximately 190,000 Specimens Examined.)

TEST RESULT	TOTAL NO.	PROBABLE DIAGNOSIS FROM AVAILABLE DATA:			
		SYPHILITIC	DOUBTFUL		NONSYPHILITIC
			WITH SUGGESTIVE DATA	WITHOUT SUGGESTIVE DATA	
Mazzini 4-plus	157	97	41	19	–
Kline negative*	18	14	4	–	–
Kahn negative†	81	54	18	9	–
C. F. negative‡	141	90	35	16	–
Mazzini 3-plus	217	145	46	26	2
Kline negative	62	49	9	4	–
Kahn negative	166	109	34	22	1
C. F. negative	207	138	43	24	2
Mazzini 2-plus	76	36	32	7	1
Kline negative	48	27	17	3	1
Kahn negative	66	31	29	5	1
C. F. negative	74	36	30	7	1
Mazzini 1-plus	11	3	4	3	1
Kline negative	2	1	1	–	–
Kahn negative	5	2	2	–	1
C. F. negative	10	3	3	3	1
Mazzini negative	41	9	9	22	1
Kline negative	4	2	1	1	–
Kahn negative	25	3	7	15	–
C. F. negative	34	5	8	20	1
Total cases	502	290	132	77	5

The tests, as performed in the Indiana State Board of Health Serological Laboratory, received the following rating in the 1938 evaluation survey conducted by the United States Public Health Service and the American Society of Clinical Pathologists:

*Kline diagnostic test: sensitivity 82.4 per cent, specificity 100 per cent.

†One tube modification of the Kahn standard test: sensitivity 82.4 per cent, specificity 100 per cent.

‡Complement fixation (Moon-Wassermann) technique: sensitivity 81.9 per cent, specificity 100 per cent.

LAKE COUNTY (INDIANA) SYPHILIS SURVEY

In the Lake County Survey Laboratory the Mazzini and the Kline diagnostic tests are done on all specimens. Kahn standard tests are done only on those which show some degree of positive reaction with the above tests. The Kahn antigen used in this series was obtained from the University of Michigan Hospital.

The tests to be presented in this series represent 10,253 blood specimens. This total number classified by test and result are shown in Table II.

TABLE II

TEST RESULT	MAZZINI	KLINE	KAHN*
4-plus	1,491	1,010	540
3-plus	402	372	256
2-plus	219	344	264
1-plus	227	444	350
Negative	7,884	7,777	839
Not done	30	306	8,004
Total	10,253	10,253	10,253

*Lest false impression is left here, please note analysis of data section concerning this table.

Since the use of the Mazzini test in the Survey Laboratory represented its first use in actual practice and since the study presented in the preliminary studies seemed to indicate that the stronger reactions were satisfactorily specific, we were anxious to guard against the possibility of syphilis being falsely diagnosed as the result of an unsupported weak positive reaction with this test and to learn more of the significance of these weak positive results. Whenever such a reaction was encountered a questionnaire was sent to the physician in charge of the case, asking whether any evidence could be found in the history or physical examination to support or to contradict the result of the serologic examination. Questionnaires were not sent on specimens marked "for treatment check." One hundred eighteen of these questionnaires were sent out; 76 (64.3 per cent) were returned. The results are analyzed in Table III.

TABLE III

CASES SELECTED AT THE SURVEY LABORATORY; SPECIAL INTEREST IN WEAK POSITIVE RESULTS OF SEROLOGIC TESTS FOR SYPHILIS

TEST RESULT	TOTAL NO.	PROBABLE DIAGNOSIS FROM AVAILABLE DATA				NO. IN GROSS DIAGNOSTIC SERIES
		SYPHILITIC	DOUBTFUL		NON-SYPHILITIC	
			WITH SUGGESTIVE DATA	WITHOUT		
Mazzini 4-plus	3	1	1	–	1	1091
Kline negative	–	–	–	–	–	
Kahn negative	1	–	1	–	–	
Mazzini 3-plus	8	5	1	2	–	283
Kline negative	–		–	–	–	
Kahn negative	5	3	1	1	–	
Mazzini 2-plus	22	9	9	4	–	162
Kline negative	6	2	3	3	–	
Kahn negative	16	5	7	4	–	
Mazzini 1-plus	43	6	14	20	3	168
Kline negative	28	4	9	14	1	
Kahn negative	35	5	11	16	3	
Total	76	21	25	26	4	9228

ANALYSIS OF DATA

Table I is largely self-explanatory; the point of chief interest is that the Mazzini technique detects more cases of syphilis than do the other tests under comparison. Particular attention is invited to the group of negative Mazzini reactions, where it is evident that this test was negative in cases which gave false positive reactions with the Kahn and Kline tests and even with the complement fixation test. Although not so clearly shown here as in the original unabridged table, the test also gave a certain number of false negative reactions which were detected by one or more of the other tests.

Table II illustrates the fact that while there is fairly close correlation between the number of negatives with the Mazzini and Kline tests (within 1 per cent of the series), the Mazzini test has taken many of the partial positives out of the doubtful range into the positive range of 3-plus and 4-plus. The apparent discrepancy between the number of positives and the number of Kahn tests done is explained partially by insufficient serum for the Kahn test and partially by the fact that for the first few weeks of operation of the Survey Laboratory, Kahn tests were not done there but positive sera were relayed to the State Board of Health Laboratory, Indianapolis, for Kahn and complement fixation tests, thus supplying a close interlaboratory check until we were assured that our results were satisfactory.

Table III is set up exactly as was Table I and represents the cases out of the series in Table II upon which questionnaires were sent out and returned; the additional column at the right indicates the total number of similar results in the diagnostic series of the gross run. Sixty-five 1-plus and 2-plus Mazzini cases represent a sampling of 19.6 per cent out of the 330 similar reactions in the gross diagnostic series. Twenty-two 2-plus Mazzini cases represent a sampling of 13.6 per cent out of 162 similar reactions in the gross diagnostic series. Forty-three 1-plus Mazzini cases represent a sampling of 25.6 per cent out of the 168 similar reactions in the gross diagnostic series. Calculation of the percentage of false positive reactions may be made according to the following formula:

$$\frac{\left(\frac{(N_s + D_s) \cdot S_G}{T_s}\right)}{T_G} = \text{Per cent of false positives,}$$

where:

N_s = nonsyphilitic cases in sample
D_s = doubtful cases in sample
T_s = total cases in sample
S_G = similar reactions in gross diagnostic series
T_G = total number in gross diagnostic series

The 2-plus Mazzini reaction was falsely positive to the extent of 1.03 per cent if all of the doubtful cases are admitted as false positives, or to the extent of 0.31 per cent if the cases classified as doubtful with suggestive data are not included. The median of this range is 0.67 per cent.

The 1-plus Mazzini reaction was falsely positive to the extent of 1.56 per cent to 0.93 per cent, or a median of 1.24 per cent.

For the combined 1-plus and 2-plus reactions, the so-called doubtful range, the per cent of false positives was from 1.24 to 2.59, with a median of 1.9. This

means that in this series the Mazzini test exhibited a specificity of approximately 98 per cent in the so-called doubtful range.

DISCUSSION

This study was undertaken primarily to increase our own knowledge of the dependability of the Mazzini test and has been pursued in an attempt to answer inquiries concerning its specificity. It is unfortunate that the data are not complete, so that the specificity for the so-called positive range of 3-plus and 4-plus might have been calculated. It logically would be much greater than in the range of 1-plus and 2-plus. The cases in Table I do not represent a true sampling, but include only those reactions which apparently were likely to reveal faults in the test. In view of this, it is believed that Table I indicates a satisfactory specificity. The calculation of specificity in the lower range is believed to be statistically sound and quite satisfactory for that range.

Specificity has been of great interest to pathologists and serologists. The clinician is more interested in what a given result, especially in the partial positive range, means in regard to his particular patient. Reference to Table III, which excludes treatment check examinations and deals only with the diagnostic series, shows that on the basis of a 2-plus Mazzini test, without knowledge of additional tests, the probability is approximately 1 in 1½ that the patient has syphilis, or 3 in 3⅔ that he presents a diagnostic problem with syphilis as a definite possibility. Similarly, the unsupported 1-plus Mazzini reaction indicates a probability of 1 in 7 that the patient has syphilis, or 1 in 2 that he presents a problem in the differential diagnosis of syphilis. Support by one or both of the additional tests definitely increases these probabilities. It was repeatedly found that only after serologic examination had shown some degree of positive reaction was a complete venereal history obtained.

It is believed that the data presented supply adequate support of our policy of reporting the various tests in terms of 4-plus, 3-plus, 2-plus, 1-plus, or negative, with the understanding that 1-plus and 2-plus are considered doubtful. The confusion of the clinician, sometimes claimed to result from such reports, should not (nor does it in our experience) for long exceed his competency.

In this analysis and discussion no attempt has been made to draw inferences concerning the indications for treatment or the continuation of it. Such a study would belong in the field of syphilology. In that respect reference is made to recent publications of Kolmer[3, 4] and Stokes.[5]

It is interesting to notice that in attempting to classify the cases of Table I as to diagnosis, difficulty was occasionally encountered, either because of a definite diagnosis of some condition other than syphilis or because of a differential diagnostic problem involving some condition other than syphilis. Such conditions included tuberculosis 8; carcinoma 7 (cervix uteri 2, liver 2, colon 1, lung 1, and carcinomatosis from teratoma of the testicle 1); pneumonia 4; hyperthyroidism 3; peptic ulcer 2; subacute bacterial endocarditis 2; Sydenham's chorea 2; mononucleosis; pneumococcic meningitis; typhus; empyema; Vincent's angina; acute rheumatic fever; staphylococcus otitis media, mastoiditis, and meningitis; and a nonpulsatile tumor in the midzone of the right chest. Incidentally, although not included in this series, "Rocky Mountain"

spotted fever has also given rise to the same difficulty in some cases. It is expected to deal further with the significance of these observations at a later time.

The serologic findings of the Lake County Survey Laboratory have been mentioned only from the standpoint of their bearing on evaluation of the Mazzini test; further analysis of their broader significance is reserved for a later study.[6]

During the past few years there has been an increased stimulus for careful scrutiny of results obtained in making serologic tests for syphilis. Increasing sensitivity has invoked vehement protests that false positives must necessarily follow. Two schools of thought have become even more sharply divided. One group believes that specificity must be safeguarded by maintaining an insensitivity of approximately 30 per cent. The other group, while recognizing the false position reaction as the paramount error of serologic technique, believes that the primary purpose of any such test is to furnish diagnostic aid in the detection of syphilis where it exists. To this end, they strive for maximum sensitivity consistent with 100 per cent specificity. The ideal is admittedly difficult to attain. In practice any test will give occasional false positives for biological reasons (that is, aside from technical faults). Therefore, in a large series of tests by any technique the specificity of 100 per cent may be approximated but not achieved. Specificity above 99 per cent for the so-called "positive" reports is, I believe, now generally accepted as satisfactory. Moreover, "all signs fail" when patients suffering from certain diseases are included in evaluation material.[2] Until more is known concerning the nature of "syphilitic" reagin, it is unlikely that we can do better than to recognize that such nonspecific results occur. This means, of course, that despite all talk of specificity the diagnosis of syphilis is, and must remain, a problem for medical judgment and must not depend entirely upon any serologic technique. This point cannot be too strongly emphasized. It would seem that a failure to appreciate this has caused undue alarm about the perils of high sensitivity.

SUMMARY

An analysis of 502 case studies (by the questionnaire method) collected in preliminary attempts to evaluate the Mazzini flocculation test for syphilis is presented. In addition, the results of the examination of 10,253 blood specimens, representing the first use of the test in actual practice, are presented. An analysis is made of a 19.6 per cent sample of the so-called doubtful reactions of the diagnostic series only. Calculated upon the basis of this sample, the test in this series maintained a specificity of approximately 98 per cent within the doubtful range. It is pointed out that within the so-called positive range of 3-plus and 4-plus, the specificity is expected to be much greater, and that the preliminary studies seem to indicate this, although calculation of this value is not attempted because of incomplete data.

The data presented indicate that the Mazzini test is more sensitive than the tests with which it is compared and is interpreted as indicative of a satisfactory specificity.

It is pointed out that a knowledge of specificity does not indicate the significance of a doubtful (or even of a positive) reaction in a given patient. An attempt is made to answer the latter question as it concerns the Mazzini test.

It is pointed out that false positives may occur with any serologic test for syphilis and that the diagnosis of syphilis should remain a problem for medical judgment.

CONCLUSIONS

The Mazzini flocculation test offers a greater aid in the serodiagnosis of syphilis than do certain other tests in common use.

Multiple tests are preferable to any single test.

Appreciation is expressed for the technical assistance of Miss Marie Martin and Mrs. Ann Walker; for the cooperation of the physicians of Indiana and especially of Lake County, without which this study could not have been made; and for the assistance of Mr. Kenneth W. Revell and his staff, of the Central Tabulating Unit of the U. S. Public Health Service, in tabulating a portion of the data.

REFERENCES

1. Mazzini, L. Y.: A Reliable, Sensitive, Simple, and Rapid Slide Flocculation Test for Syphilis, Am. J. Clin. Path. **9**: 163, 1939.
2. Cumming, H. S., Hazen, H. H., Sanford, A. H., Senear, F. E., Simpson, W. M., and Vonderlehr, R. A.: The Evaluation of Serodiagnostic Tests for Syphilis in the United States: Report of Results, J. A. M. A. **104**: 2083, 1935.
3. Kolmer, J. A.: Serologic Reactions and Immunity in Relation to Infection and Treatment of Syphilis, Am. J. Syph., Gonor. & Ven. Dis. **22**: 426, 1938.
4. Kolmer, J. A.: Laws Requiring Premarital and Pregnancy Tests for Syphilis, J. A. M. A. **112**: 2385, 1939.
5. Stokes, J. H.: Some Problems in the Control of Syphilis as a Disease, Am. J. Syph., Gonor. & Ven. Dis. **23**: 549, 1939.
6. Niblick, J. S., Ratcliffe, A. W., and Shanklin, E. M.: The Lake County Syphilis Survey, J. Ind. St. Med. Assoc. **33**: 318, 1940.

DEPARTMENT OF REVIEWS AND ABSTRACTS

ROBERT A. KILDUFFE, M.D., ABSTRACT EDITOR

SULFANILAMIDE, Studies on the Mechanism of the Action of, Lockwood, J. S., and Lynch, H. J. A. M. A. **114**: 935, 1940.

Sulfanilamide has a bacteriostatic and limited bactericidal action in vitro on hemolytic streptococci, staphylococci, pneumococci, and colon bacilli. The magnitude of this effect is dependent principally on (a) the concentration of the drug, and (b) the concentration of "peptone" in the culture media.

"Peptone," as used in this paper, connotes any product of protein digestion whether prepared artificially in vitro or through the operation of natural enzymatic processes in vivo.

It is the author's belief that sulfanilamide acts by interfering with the nutritional requirements of susceptible bacteria and that the bacteria can die out in a phagocyte-free environment through starvation and autolysis.

The addition of peptone to media such as serum, which are deficient in nitrogen easily assimilable by bacteria, supplies such an excess of nitrogenous material that the bacteriostatic action of sulfanilamide is to a large degree inhibited.

JAUNDICE, Obstructive, The Renal Lesion in, Thompson, L. L., Frazier, W. D., and Ravdin, I. S. Am. J. M. Sc. **119**: 305, 1940.

Except for the fact that their patients had a high incidence of albuminuria and a number of high blood urea nitrogen figures, the authors' clinical and experimental studies strongly confirm Elsom's observations on renal changes in hepatic disease.

A pre-existing renal lesion is intensified by a secondary "cholemic nephrosis." The high incidence of glomerular changes in the patients that died suggests that a pre-existing renal lesion may have been present. This is strongly suggested by the fact that these changes predominated in elderly patients.

If no previous renal injury exists, the evidences of "cholemic nephrosis" disappear rapidly following release of the obstruction. The major injury in this disorder is confined to the tubules.

The nephrosis associated with bile duct obstruction may be a determining factor in the final outcome and should not be looked upon as an inconsequential complication.

VITAMIN C, Effect of Renal Retention of, on Saturation Tests, Ludden, J. B., and Wright, I. Arch. Int. Med. **65**: 151, 1940.

A modified five-hour vitamin C saturation test to compensate for the error due to variations in renal function is proposed.

Twenty-four-hour studies of the urinary excretion and plasma concentration of vitamin C after an intravenous test dose of 1 Gm. of ascorbic acid are reported for patients with a wide range of saturation levels and with various degrees of renal insufficiency.

Except for patients in the uremic state, the twenty-four-hour excretion appears to give a satisfactory index of the state of vitamin C nutrition. The excretion during shorter test periods gives false low values for patients with retarded vitamin C excretion. Patients with impaired renal excretion of vitamin C may give no other evidence of renal insufficiency.

A correlation exists between (1) the percentage of the five-hour output excreted during the first one and one-half hours after the test dose, and (2) the percentage of the

twenty-four-hour output excreted during the first five hours. In view of this, a formula is presented by which the actual twenty-four-hour output may be satisfactorily predicted through the use of data on urine specimens obtained one and one-half and five hours after the test dose.

The proposed test, in experience, gives a more reliable estimation of the actual state of vitamin C saturation than any other method available at this time.

In addition, an estimation of the renal function for vitamin C may be obtained through the analysis of data from this test.

The methods used follow:

Each subject was placed on a diet containing a minimum of vitamin C (less than 15 mg. daily) for at least twelve hours preceding the test and for twenty-four hours after the administration of the test dose. At 9 o'clock on the morning of the test the patient voided; six cubic centimeters of blood were drawn for plasma analysis and 1 Gm. of ascorbic acid, dissolved in 10 c.c. of physiologic solution of sodium chloride, were injected intravenously. Complete specimens of urine were obtained exactly one and one-half, three, and five hours after the injection, and were titrated immediately. The remaining specimen (nineteen-hour) was collected in tightly stoppered dark brown bottles, containing sufficient glacial acetic acid to bring the pH to 3, and kept at icebox temperature. This specimen was titrated immediately after the last micturition. For a number of patients with normal vitamin C excretion and for all patients with delayed excretion, the specimen obtained on the twenty-fourth hour was collected and titrated apart from the rest of the nineteen-hour specimen; blood was obtained from these patients at periods corresponding with the urine collections, and the plasma was analyzed for vitamin C.

The vitamin C in the urine was determined by a modification of Tillman's 2,6-dichlorophenolindophenol method and in the plasma by the sodium tungstate precipitation method of Farmer and Abt. (Duplicate plasma determinations, using the metaphosphoric acid technique of Farmer and Abt, gave values that agreed within 0.04 mg. per hundred cubic centimeters.) In every instance the plasma analysis was done within thirty minutes after venipuncture, and suitable blanks were used in all titrations. Potassium cyanide was not used as a preservative, since previous studies have shown that it reduces the dye and produces erroneous values.

The following modification is suggested for the saturation test:

Have the patient omit breakfast on the morning of the test. Immediately after the patient has voided and discarded the preliminary urine, inject intravenously 1 Gm. of ascorbic acid dissolved in 10 c.c. of physiologic solution of sodium chloride. Collect urine specimen 1 exactly one and one-half hours after the injection, and specimen 2 exactly five hours after the injection. Titration should be done preferably immediately after each collection, but, if necessary, the first specimen may be preserved as outlined previously and titrated at the end of the test.

By means of the values obtained for specimen 1 (a) and the sum of those for specimens 1 and 2 (b), the predicted twenty-four-hour excretion, C, may be calculated according to the formula $C = \frac{ab}{12.6a - 0.27b}$. The value thus calculated may be termed the saturation index.

RESPIRATORY TRACT, An Epidemic Disease of, Reimann, H. A., and Havens, W. P. Arch. Int. Med. **65**: 138, 1940.

An epidemic disease of the respiratory tract occurred in Philadelphia and elsewhere in the winter of 1939. Reports of similar outbreaks in many parts of this country and in Europe suggest that it may have been pandemic. Of a group of 813 persons in the personnel of the Jefferson Medical College and Hospital, 50 per cent were ill. The majority of patients (88 per cent) were ill with nasopharyngolaryngitis. Six per cent had tracheobronchitis in addition, and 6 per cent had tracheobronchopneumonia. In most of the severely ill patients the lungs were presumably involved by the infectious agent suspected without the agency of the usual varieties of bacteria. There were no serious complications,

and all the patients recovered. The disease resembles epidemic influenza in many respects, but it is caused by a different agent. It represents a clinical entity probably caused by a filtrable virus.

SULFAPYRIDINE, Parenteral Administration of, Haviland, J. W., and Blake, F. G. Am. J. M. Sc. **119**: 385, 1940.

The authors have presented a method of administering sulfapyridine parenterally which they deem quite satisfactory. One disadvantage of the method might appear to be the relatively large volume of fluid which must be used in order to put the desired quantity of sulfapyridine into solution. On the other hand, the authors have demonstrated by actual trial that such volumes may be given safely to almost any patient when necessary. The method will maintain adequate therapeutic blood levels. It will provide adequate fluids, salt, and glucose at a time when they are often needed either because of persisting vomiting or inadequate intake in delirious or comatose patients. It can be given without fear of slough or other reactions, such as may follow the use of the extremely alkaline sodium sulfapyridine. Furthermore, it may be given into any part of the body where needed, such as the veins, the subcutaneous tissue, the pleural cavities, or the subarachnoid space.

The method follows:

A weighed amount of the powdered drug was poured into the desired volume of the liquid to be tested or used. This liquid had been brought to the boiling point just before the addition of the powder. The mixture was shaken vigorously until all the powder had gone into solution, usually for one or two minutes. (Occasionally it seemed expedient to heat the mixture again to the boiling point in order to facilitate complete solubility.) The solution was then allowed to cool for six to eight hours, or until it had reached room temperature. Portions of these mixtures were then analyzed for their sulfapyridine content. Therapeutically the solutions were generally cooled fairly rapidly to the desired temperature.

The solutions were observed to assume a pale golden color upon standing. Whenever sulfapyridine precipitated out, it seemed to carry more and more of the substance with it, so that after a day or two a considerable clump of powder could be visualized on the bottom of the flasks. However, when the solution was complete, the sulfapyridine remained dissolved for several days. Aerobic and anaerobic cultures of such fluids were found to be sterile.

TRICHINOSIS, A Study of 23 Cases, Murphy, F. D., James, H. D., and Rastetter, J. W. Am. J. M. Sc. **119**: 328, 1940.

The following points summarize the safeguards that the authors believe should be used for the control of trichinosis.

1. Individuals by their food habits expose themselves to the disease; the public should, therefore, be informed of the danger of trichinosis. They should be taught emphatically that insufficiently cooked food containing any pork is dangerous. The consumer must be brought to realize that he is, in the last analysis, responsible for getting trichinosis.

2. As Pote points out, the least likely source of the parasite is pork products prepared under Federal and adequate municipal supervision. The authors agree with his theory that it is the unprocessed pork products, especially summer sausage, prepared without supervision in small local slaughter houses or on farms, that frequently cause trichinosis. The jurisdiction of public health organizations should be made to include these sources of pork products.

3. The packer and swine grower, as pointed out by Hall, have it within their power and should be given the responsibility of setting up safeguards against the consumption of infested pork, particularly by excluding the hog which is fed uncooked pork scraps.

4. The medical profession should keep in mind the possibility of the disease when unusual cases of "flu" or "grippe" do not respond readily to treatment. If the physician

suspects the disease, the diagnosis usually will be made readily. All cases should be reported to the health department and attempts made to discover the source of the parasite.

Finally, it is emphasized that while the immediate responsibility for contracting trichinosis rests upon the consumer, the United States Public Health Service should assume the chief role in protecting the public from this disease.

IODINE, Blood, in Acne Vulgaris, Traub, E. F., and Emmet, R. Arch. Dermat. & Syph. **41**: 506, 1940.

A series of 58 patients were studied from the standpoint of the iodine content of the blood.

Forty-five patients suffered from acne vulgaris. Thirteen patients had miscellaneous cutaneous diseases and were used as controls. Twenty-two additional, apparently normal persons, not included in the afore-mentioned group of 58, were also used as controls.

In the control groups of patients, whose blood iodine was apparently normal, the average value was approximately 6 micrograms per hundred cubic centimeters. The average iodine content of the blood of the patients with acne vulgaris was 5.975 micrograms, which was approximately within the normal range.

A slight difference was noted between the iodine content of the blood of males and that of females, the values for the males averaging 5.3 micrograms per hundred cubic centimeters and those for the females 6.65 micrograms.

Differences in the iodine content of the blood of patients in various age groups were recorded.

The failure to find a higher blood iodine content in patients with acne vulgaris than in "normal" persons possibly indicates that (1) the role, if any, played by iodine may be qualitative or (2) the iodine may, in patients with this disease, be stored in the cutaneous tissues.

MEASLES, Disseminated Giant Cell Reaction, a Possible Prodrome of, Stryker, W. A. Am. J. Dis. Child. **59**: 468, 1940.

A pathologic change in a 27-month-old child who died of pneumonia was the presence of multinucleate giant cells in the pulmonary alveoli, bronchi, sinuses, and medullary cords of the tracheobronchial lymph nodes, lumina, and walls of the bronchial mucous glands, interstitial connective tissues about the lymph nodes and mucous glands, spleen, and lymphatic tissues of the ileum. These cells appeared morphologically like those described as specific for the prodromal stage of measles. A history of a possible contact with measles was obtained. Attempts to demonstrate inclusion bodies were inconclusive. This case is not presented as a proved example of the appearance of disseminated giant cells in the prodromal stage of measles; the existence of similar cells in proved cases, however, suggests the possibility here.